Clinical and analytical aspects of the improvement of a local antifungal preparation for the treatment of nasal polyposis

Ph.D. dissertation

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I. List of Contents

II. Abbreviations

AmB	amphotericin B
ARS	adherence risk scale
BMQ	brief medication questionnaire
CRS	chronic rhinosinusitis
CRSwNP	chronic rhinosinusitis with nasal polyposis
CT	computer tomography
DMSO	dimethyl sulfoxide
EM	eosinophilic mucin
FESS	functional endoscopic sinus surgery
HPLC	high performance liquid chromatography
HPTLC	high performance thin layer chromatography
LOD	limit of detection
LOQ	limit of quantification
MIC	minimal inhibitory concentration
NP	nasal polyposis
Ph.Eur.6.	European Pharmacopoeia 6th edition
SD	standard deviation
RSD	relative standard deviation
USP.21.	United States Pharmacopoeia 21st edition

III. General introduction and aims

High quality pilot clinical studies occupy an important role in the development of novel medications and also in gathering information of diseases with yet unknown or debated etiological background. Especially in such cases when relatively rare or orphan diseases are in the scope of research, since patients suffering from these illnesses can hardly hope for large pharmaceutical companies (controlled by the rules of economy and competition) to develop medications for their needs. Although pilot clinical studies have the disadvantage of including only smaller amount of patients, with the meta-analysis or systematic review of several publications good, comprehensive evidence can be gathered on a certain topic.

It has to be stated, that a well organized and planned study requires thorough preparation and also the collaboration of different healthcare professionals (medical doctors, pharmacists, nurses, microbiologists, statisticians, analytical etc.). Each profession gives an added value to the work and is essential for the successful completion of the study.

As a pharmacist I would like to highlight some important topics regarding a pilot clinical study I had a chance to participate in. I hope that my work gives a good example of the diversified professional challenges that clinical pharmacists have to face.

In 2006 a double blind, randomized pilot clinical study was planned and launched to assess the efficacy of long term intranasal antifungal treatment in patients suffering from chronic rhinosinusitis with nasal polyposis (CRSwNP), because it has been hypothesized that fungal cells in the mucus exacerbate adverse immune response that result in the formation of polyps. Several studies have been published on the issue and controversial results can be found on the effectiveness of antifungal treatment. Before this well prepared study was started, the question of stability arose regarding the study medications. After the completion of a three month stability test further questions had to be faced regarding the adequate analysis of amphotericin B (AmB) solutions. Instructive and useful experience was gained while problems of microbiologic and instrumental analytical possibilities were solved.

In my thesis I will aim to introduce a complex pharmaceutical view regarding a pilot clinical study organized by the Department of Otorhinolaryngology and Head and Neck Surgery in cooperation with Department of Pharmaceutics and University Pharmacy of the

University of Pécs in 2006. During the preparation phase we had to face several pharmaceutically important questions.

The aims of my thesis are to:

- evaluate earlier clinical studies regarding to the antifungal treatment for chronic rhinosinusitis;
- assess the efficacy of long-term AmB treatment in patients with CRSwNP and also to describe the compliance of patients in the study;
- discuss pharmaceutically important topics regarding the study sample, such as the preparation of clinical samples, optimal dosage form, proper concentration of the active ingredient, storage and stability;
- introduce the different chemical and microbiological analytical methods for the measurement of AmB and summarize our work in the improvement of these techniques.

Since such diversified topics and fields of research are included in my work, in the interest of transparency I divided the thesis into two sections. In the first clinical section the etiology and the therapy of CRSwNP is discussed along with the detailed presentation of the double blind pilot study of Pécs and the results of our compliance measurements. In the second analytical section the challenging problem of the analysis of AmB and the questions regarding a stability testing are introduced. Also the applied chemical and biological methods are presented and compared.

IV. Section One: Clinical part

A. Chronic Rhinosinusitis with Nasal Polyposis

Chronic rhinosinusitis (CRS) is an inflammatory disease of the nasal and paranasal sinus mucosa that has existed for more than 3 months, with typical leading symptoms such as nasal obstruction, thick mucopurulent nasal discharge, a reduction/loss of the ability to smell, facial pressure and/or pain, in some cases accompanied by an extreme degree of nasal polyposis (NP) (1). As concomitant minor symptoms, CRS patients may complain of headaches, fever, halitosis, fatigue, dental pain and ear fullness.

1. Epidemiology of the disease

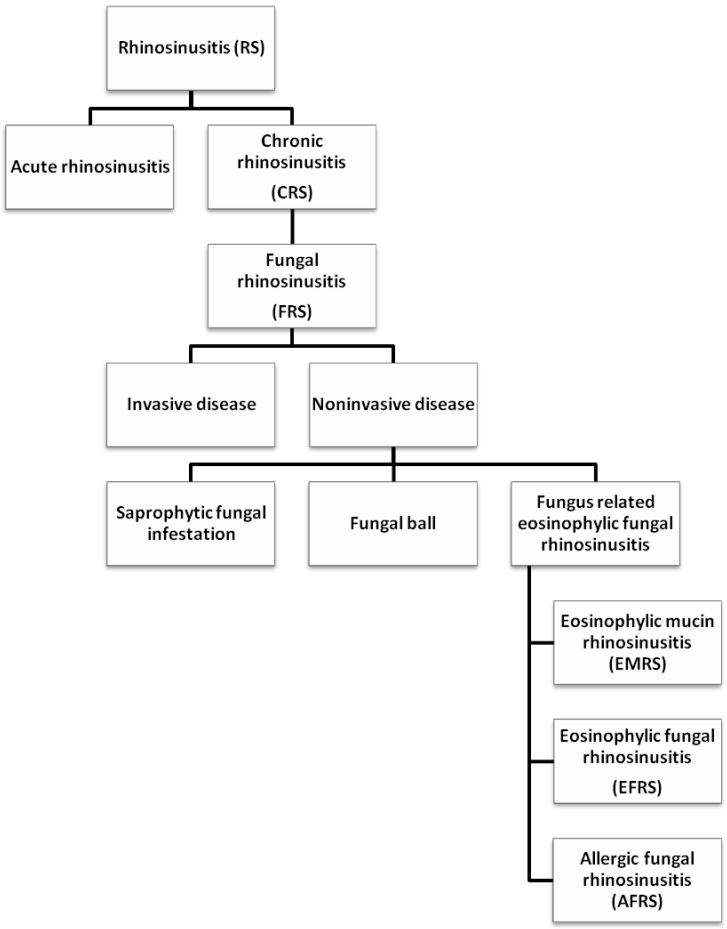
Current medical research suggests that CRS can be referred to as a multifactorial disease, which may be associated with asthma, cystic fibrosis, primary ciliary dyskinesia, aspirin intolerance and allergy (2). Around 4% of the population suffers from a compromised quality of life in consequence of the disease (3). It affects mainly adult patients older than 20, with at least 2:1 male to female ratio. One third of patients with NP have asthma, but polyps are found in only 7 % of asthmatics (4). Significant medical resource expenditures and global economic costs accompany CRS (5).

2. Etiology and the theories of polyp development

The etiology and pathogenesis of CRS are neither largely unknown. One of the most popular theories, which is a subject of intensive research, postulates that the causal factors are morphological variations on the lateral wall of the nasal cavity (3); other hypotheses include the biofilm theory (6) and the role of superantigens (7). The basic standard treatment is surgical intervention and the use of corticosteroids. It should be emphasized that even the most modern antibiotics are effective only during the acute exacerbations of CRS, making a bacterial etiology doubtful. The fact that antiallergic medication results in a symptomatic

improvement merely in the event of a proved concomitant allergy suggests that an IgE-mediated allergy is not an etiological factor of CRS (8).

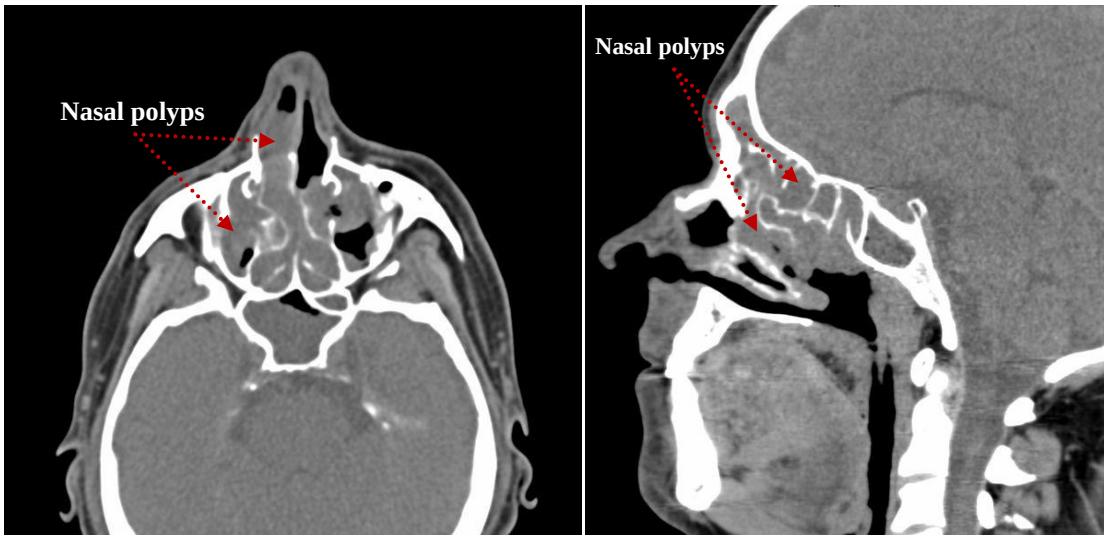
Figure 1. Categorization of rhinosinusitis and current classification of fungal rhinosinusitis (9)**.**



The role of fungal organisms in the development of some rare forms of CRS has long been known. Allergic fungal rhinosinusitis (AFRS) was first described by Katzenstein et al. in 1980 (10). DeShazo and Swain (11) and later on Bent and Kuhn (12) proposed the criteria of this previously rarely diagnosed disease. Ponikau et al. recently developed new mucus-collecting and culturing methods with which they were able to demonstrate the presence of mucin containing hyphae and clusters of degenerating eosinophils (Charcot-Leyden crystals) referring to allergy in 96% of CRS patients with polyposis undergoing endoscopic sinus surgery (13). See Appendix A for the list of cultured fungi from patients and healthy adults. With the diagnostic criteria of DeShazo and Swain, the previously rarely diagnosed AFRS was seen in 93% of their patients. To their surprise, with this new method they cultured at

least two fungal species individually in the nasal mucus of a control group consisting of 14 healthy adults, but without detection of the presence of an IgE-mediated type I hypersensitivity reaction. The number of organisms recovered from patients and healthy control subjects are summarized in Appendix A. In the opinion of Ponikau et al. "allergic mucin" exists independently from an IgE-mediated type I hypersensitivity reaction. For this reason, they proposed a change in the terminology: they prefer the term "eosinophilic mucin" (EM) instead of "allergic mucin", and suggest that "allergic fungal rhinosinusitis" be changed to "eosinophilic fungal rhinosinusitis". Thus, the eosinophilic granulocytes within the nasal mucus of CRS patients do not play a part in allergic reactions, but are transitory components of the nasal secretion. After destroying the hyphae, they fall apart and the major basic protein released from them exerts an extremely toxic effect on nasal mucosa (14). As a consequence of secondary superinfection of the nasal mucosa via an epithelial lesion, biofilm colonization and the appearance of superantigens CRS may develop. All these facts indicate the multifactorial nature of the disease.

Figure 2. Computer tomography scans (axial view on the left, side view on the right) of a patient with intensive polyposis obliterating the ethmoid- , frontal , maxillary and sphenoid sinuses and the nasal cavities.



Pant et al. recently proved the absence of fungi in the EM of some CRS patients (15). They also demonstrated the similarity in clinicopathologic features between EM CRS subgroups despite the occurrence of positive fungal cultures and hypersensitivity against fungi. Their observations indicated that the presence of EM always reflects a more severe grade of CRS than that in its absence. Erbek et al. showed that the grade of eosinophilia is

more influential as regards the severity of CRS than the presence of a positive fungal culture (16).

If the fungal theory holds true, it seems obvious that, through a reduction of the amount of antigen, or its total eradication, thereby influencing the triggers of CRS, the symptoms of the patients can be relieved. Accordingly the treatment of CRS patients with intranasal lavage or a spray containing AmB would be beneficial.

B. Review and evaluation of earlier publications

1. Introduction of studies

During the past 7 years, seven clinical studies have been published in which experience with local antifungal treatment regimes in CRS was discussed (13, 17, 18, 19, 20, 21, 22). The controversial characteristics of these studies are summarized in **Table 1**. Only three of the papers were based on double-blind, placebo-controlled studies (18, 19, 22), and of these three studies only one was multicentre (22), making the interpretation of the results rather difficult. One of the studies excluded patients with AFRS completely (19). In some of the studies, the number of recruited patients was extremely low (20, 18). The treatment period ranged between 4 and 80 weeks, and the form of drug/placebo application also varied since nasal lavage, nasal spray and nasal inhalation was used. The concentration and the daily amount of AmB recommended also differed in these studies. The conditions of drug storage and checking the stability of the AmB solutions was completely ignored in most of these studies. Due to the fact that the patient compliance was examined in only one study (22), it is extremely difficult to draw conclusions concerning the effectiveness or ineffectiveness of AmB treatment.

Recently a multicenter randomized controlled study has been published evaluating the effect of topical AmB on inflammatory markers in samples of nasal lavage fluid, from patients with CRS with or without NP. The authors had to conclude that AmB had no significant effect on the level of any of the tested pro-inflammatory cytokines, chemokines and growth factors (23).

Table 1. Summary of clinical studies of amphotericin B treatment of Chronic Rhinosinusits with Nasal Polyposis

Authors	A. Ricchetti et al.	J. U. Ponikau et al.	J. U. Ponikau et al.	M. Weschta et al.	A. Helbling et al.	C. Corradini et al.	F. A. Ebbens et al.
Year	2002	2002	2005	2004	2006	2006	2006
Journal	J Laryng & Otol	J Allergy Clin Immunol	J Allergy Clin Immunol	J Allergy Clin Immunol	J Laryng & Otol	J Investig Allergol Clin Immunol	J Allergy Clin Immunol
Type of study	open	open	double-blinded, placebo-controlled	double-blinded, placebo-controlled	open	open	multicentre, double-blinded, placebo controlled
Number of patients involved	74	51	30	78	21	89	116
Amphotericin B treatment	74	51	10	28	21	39	59
Length of treatment	4 weeks	12 weeks	24 weeks	8 weeks	12 weeks	80 weeks	12 weeks
Application	lavage	lavage	lavage	spray	spray	inhalation	lavage
Amphotericin B concentration	0,1 mg/ml	0,1 mg/ml	0,25 mg/ml	3 mg/ml	10 mg/ml	3,33 mg/ml	0,1 mg/ml
Daily amount of Amphotericin B	8 mg	8 mg	20 mg	4,8 mg	3 mg	0,8 and 0,5 mg	10 mg
Daily application	2x20-20 ml	2x20-20 ml	2x20-20 ml	4x0,2-0,2 ml	3x0,1-0,1 ml	1x0,24/0,16 ml	2x25-25 ml
Storage mentioned	no	no	no	refrigerator	refrigerator	no	refrigerator
Stability mentioned	no	no	no	no	no	no	yes
Compliance mentioned	no	no	no	no	no	no	yes
Final outcome	**Effective**	**Effective**	**Effective**	**Ineffective**	**Ineffective**	**Effective**	**Ineffective**

2. Controversial and ignored factors in previous studies

There are several factors which need to be evaluated and discussed in detail. These are the following:

- Dosage form/application method of sprays and nasal lavages
- Preparation of spray solutions and concentration of the active substance
- Role of added glucose in the solutions
- Length of therapy
- Compliance of patients
- Storage and stability of study medication

In the previously published studies different dosage forms were used. In case of nasal lavages/irrigation the sinuses are washed with 20-25 ml solution. It is arguable, whether the washing effect of the solution could in itself be beneficial in CRS (24), in this way potentially influencing therapeutic outcome.

In general if the concentration of the antifungal substance is higher in the solutions than the minimal inhibitory concentration (MIC) for a specific yeast or filamentous fungi, presumably it would be effective. It should be taken into consideration that because of ciliary activity in the nose, the active ingredient would be diluted. The antifungal spectrum of AmB is particularly broad and the great majority of fungi are susceptible to a very low concentration, generally at a MIC of < 0,5 μg/ml (25). In previous studies the concentration of solutions ranged from 0,1 to 10,0 mg/ml, in all cases exceeding MIC by 2000-20000 fold.

The substance AmB is insoluble in water. Although it can be dissolved in dimethyl sulfoxide (DMSO) such solutions cannot be used as nasal sprays since the solvent would irritate the nasal mucosa. Thus previous AmB sprays and washes for clinical studies were made from commercially available preparations such as Fungizone and Amphomoronal. These are colloidal suspensions with bad stability parameters and according to the manufacturer's instructions the water base solutions can't be diluted with saline, because monovalent ions would cause the precipitation in the colloidal system. Because it has been documented that during intravenous administration any precipitation increases the toxicity, only 5% glucose solution is considered to be sufficient for the dilution of AmB colloidal

preparations (26). Presumably for this reason one author used glucose infusions for the dilution of nasal spray solutions (19). In our judgment glucose could adversely influence the antifungal effect of medications since it is well known that glucose accelerates the growth of fungi and act as an energy source for most medically important fungi.

It should be noted that according to Meltzer (27) the antifungal treatment should last for at least 6 months and only three publications fulfil this criteria.

The former authors have not referred to any published *stability* tests of AmB solutions, thus the constant antifungal activity of the test medications between visits was questionable in each case. Although AmB solutions are considered to be relatively unstable, only three authors have referred to optimal *storage temperature.*

More than 450 patients were included in these studies, but the attitude of the patients and their *adherence to the medication* has not been reported.

In an attempt to clarify the situation, we conducted a double-blind, prospective, randomized, placebo-controlled clinical study. This differed from the previous ones in that, the active drug or placebo was administered postoperatively in the form of a nasal spray. We preferred the use of a nasal spray to nasal lavage because of the well-known, favourable effect of hypertonic saline solution on the symptoms of CRS (28, 29). As a result of nasal lavage, the mucociliary activity improves, the degree of mucous membrane oedema and the concentrations of inflammatory mediators decrease and the inspissated mucous can be cleared mechanically (30).

It is important from the aspect of compliance that application of a nasal spray is more convenient for patients than either nasal lavage or inhalation. A significant amount of nasal lavage is frequently swallowed, questioning its therapeutic effectiveness in the depths of polyp-filled sinuses, especially if it is used preoperatively. The application of AmB is favoured by the facts, that the drug is not/or poorly absorbed through the gastrointestinal tract, and it is highly effective (90%) against the majority of fungi cultured from the sinonasal tract (see cultured fungi in **Appendix A).** In our study the concentration of the drug was 5 mg/ml, which is at least 1600-5000 times higher than the MIC. We considered it reasonable to apply the AmB in the postoperative period, when the drug could pass easily and effectively into the EM-free sinuses.

C. Results of the randomized, double blind placebo control study at Pécs

1. Patients and methods

In a double-blind, randomized, placebo-controlled study, patients received AmB (A group) or placebo (B group) nasal sprays for 12 months after polypectomy with functional endoscopic sinus surgery (FESS). Our aim was to determine whether any difference could be observed between the two groups in the rates of recurrence of nasal polyposis, in the symptoms, in the quality of life or in the endoscopic findings. The protocol of the study was approved (no. 2005/11.25/2632) by the Regional Ethics Committee of the University of Pécs Medical Centre. All the patients received concomitant nasal steroid sprays and were allowed to use their regular medication. The study protocol is presented in **Figure 3.** Thirty-three patients with CRSwNP were recruited from among the patients presenting at our clinic for endoscopic sinus surgery between November 2005 and October 2006. Thirty patients completed the study; 3 patients were excluded (group A: 2, group B: 1) because of noncompliant behaviour. The patient demographics are shown in **Table 2.**

Figure 3. Study protocol of the double blind placebo control clinical study

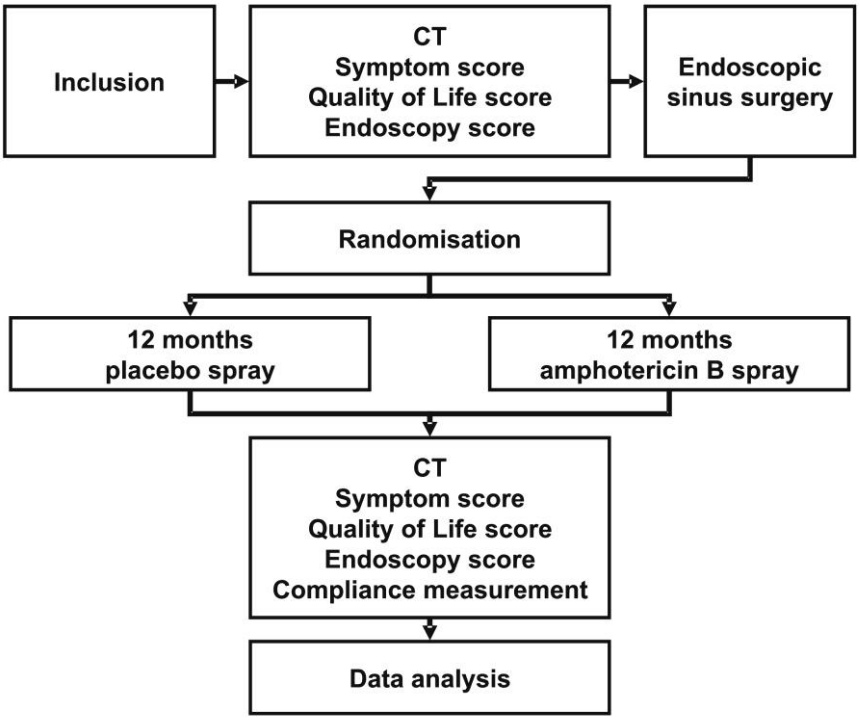


Table 2. Demographic data on patients participating in the clinical study

	Amphotericin B group (*n* = 14)	**Placebo group (*n* = 16)**
Mean age (SD)	51 (10.32)	56 (9.81)
Male/female	9/5	10/6
Asthma *(n)*	3	5
ASA intolerance (*n*)	1	1
Allergy (general) (*n*)	6	8
Allergy (antifungal) (*n*)	0	0
Smoking (*n*)	3	3
Previous procedures		
FESS (1 op)	3	3
FESS (2 op)	0	1

The diagnosis for CRS was set up according to the criteria laid down by the "EAACI position paper on rhinosinusitis and nasal polyps, executive summary" (3). The diagnosis was confirmed by the presence of symptoms that has existed for 3 months and the swelling of the nasal mucosa by 5 mm in at least 2 sinuses according to the CT scans and endoscopic observations. Exclusion criteria are listed in **Table 3.**

Table 3. Criteria for exclusion from the clinical study

Known hypersensitivity to amphotericin B
Pregnancy or lactation
Age <18 years
Suspicion of allergic fungal rhinosinusitis
Mental disease, cystic fibrosis
Osteoporosis, chronic liver or renal disease
Immune-compromised state (HIV, transplantation, diabetes)
Acute upper airway infection (within a week)
Complication of CRS (e.g., abscess)
Acute bacterial exacerbation of CRS (acute pain, pressure feeling, high temperature, mucopurulent discharge)
Orbital or intracranial complication of CRS
Antibiotic and/or antihistamine therapy within 3 weeks
Application of oral steroid within 3 weeks
Systemic antifungal therapy within 1 week

Patients were assigned to the two treatment arms by simple randomization. The AmB and placebo nasal sprays were compounded in the Central Clinical Pharmacy of Pécs. The two solutions were indistinguishable by colour, smell or taste. The active spray made from Fungizone (Bristol-Myers Squibb, Epernon, France) contained 5 mg/ml AmB, 4.1 mg/ml sodium deoxycholate and 20.2 mg/ml sodium phosphate in sterile distilled water, while the placebo was an aqueous 0.2 μg/ml acriflavin chloride solution. The antifungal activity of acriflavin chloride was assessed earlier and proved to be ineffective against the tested fungus strain (*C. albicans*) even at hundred fold higher concentrations. The sprays were manufactured under aseptic conditions and were measured out in brown, light-rejecting glass containers with dosing spray caps. The patients received 7 spray containers of 3.3 ml solutions monthly and were instructed to apply 2 times 2 doses (100 μl) daily into each nostril, i.e. the total daily dose of AmB was 4 mg/day. We earlier tested the stability of the solutions at 4 °C: the 5 mg/ml AmB solutions proved stable for 30 days (31)(see also *Chapter VI.C.*).

Primary outcome variables

Modified Lund-Mackay CT score

The scoring system as modified by Juniper (32, 33) was used to evaluate the computer tomography (CT) scans of the patients prior to endoscopic sinus surgery and also after the 12-month treatment period (*see* **Appendix B1**.).

Secondary outcome variables

Sinonasal Assessment Questionnaire (SNAQ-11)

Compared with similar tools (the Sino Nasal Outcome Test 20, SNOT-20; or the General Nasal Patient Inventory, GNPI) the 11-item SNAQ-11 (34) allows a more adequate evaluation of the main symptoms of CRS (e. g. nasal congestion or loss of the sense of smell), without a deep analysis of the nonspecific symptoms (such as a feeling of aural pressure). The questionnaire is shown in **Appendix B2**. The questionnaires were completed by all the patients both before surgery and one year later, at the end of the study period.

Quality of life test

The questionnaires were filled out prior to surgery and after 12 months (**Appendix B3**.). The patients answered the 6 questions on a 7 grade scale (35); the maximum score was 36.

Endoscopic assessment

Following nasal mucus membrane congestion all the patients were graded preoperatively and one year later, at the end of the therapy, according to the scoring system of Malm (36). All grading was performed by the same MD. The scores ranged from 0 (no polyp) to 3 (total obstruction).

Statistical analysis

For the analysis of the primary outcome measure (the CT score), the SNAQ-11 and the quality of life scores, independent sample t-tests were used. The applicability of this was checked with the Levene test, and the normality of the variables was demonstrated with Kolgomorov-Smirnov test. The procession and evaluation of the data were performed with the SPSS 14.0 for Windows (SPSS Inc., Chicago, Illinois, USA). The results are expressed as medians ± interquartile ranges.

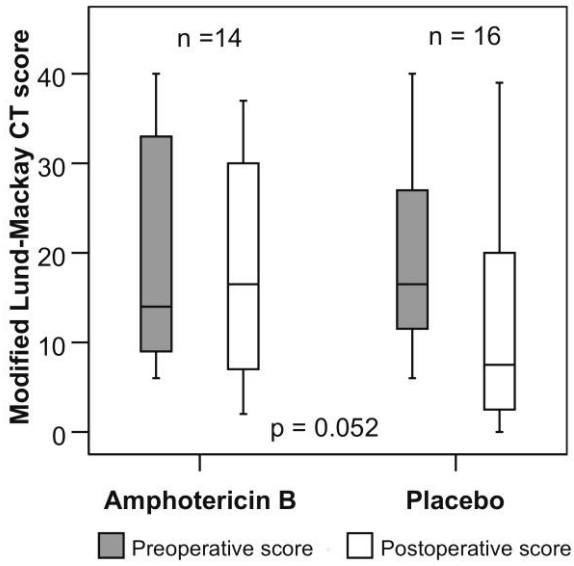
2. Results

Fourteen of the 16 patients in group A, and 16 of the 17 patients in group B completed the study.

Modified Lund-Mackay CT score

During the evaluation of the preoperative and postoperative CT scans, we observed high standard deviation in the scores without any relevant improvement. Though a slight improvement was seen in the placebo group (**Figure 4**.), the comparison of the two patient groups did not indicate a significant change (p=0.052).

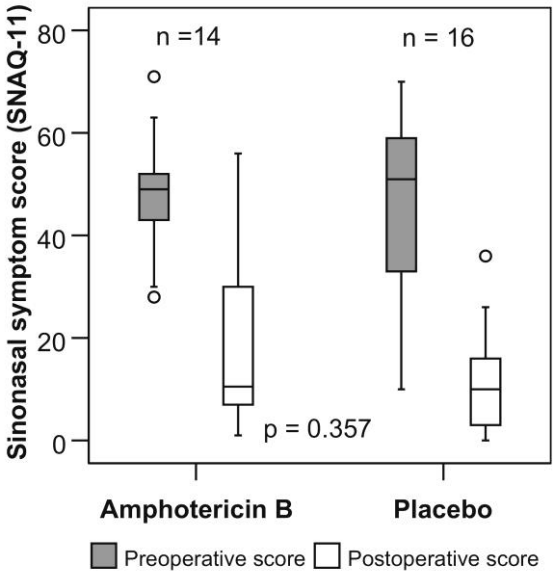
Figure 4. Preoperative and postoperative CT scores (modified Lund-Mackay test) in the two groups of patients. Means are indicated by horizontal lines, the rectangle denotes the mean ±25 % values.



Sinonasal symptom score (SNAQ-11)

A relevant improvement in the symptoms was observed in both medication groups (**Figure 5.**) but a statistically significant difference was not observed when the changes in the two groups were compared (p > 0.1).

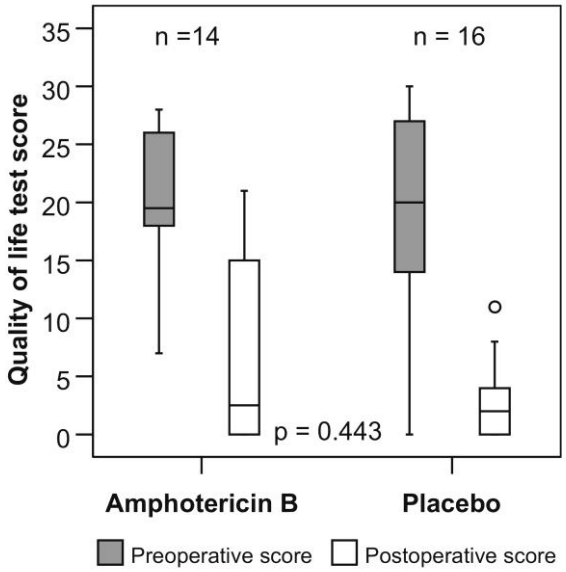
Figure 5. Preoperative and postoperative symptomatic scores in the two groups of patients



Quality of life test

A definite improvement was observed in both groups after 12 months (**Figure 6**.), but the rates of improvement in the two groups did not differ statistically significantly ($p > 0.1$). Overall, the changes in the sinonasal symptoms and quality of life scores correlated with each other but a statistically significant change was not observed in the rate of improvement between the two groups.

Figure 6. Preoperative and 12 month postoperative quality of life test data in the two groups of patients



Endoscopic assessment

Table 4. shows the endoscopic status of the patients at the beginning and at the end of the study, based on the system of Malm. Although the study population was relatively small, it can be clearly seen that there were more patients in the placebo group whose endoscopic status did not change. On the other hand, 1, 2 and 3-level changes were observed in more cases in the AmB group.

Table 4. Preoperative and 12-month postoperative endoscopic stages in the two groups of patients

Endoscopic stages		**Amphotericin B**	**Placebo**
Preoperative	Postoperative	**group (n)**	**group (n)**
Stage III.	Stage III.	0	0
Stage III.	Stage II.	1	2
Stage III.	Stage I.	2	2
Stage III.	Stage 0.	4	2
Stage II.	Stage II.	1	1
Stage II.	Stage I.	2	2
Stage II.	Stage 0.	2	2
Stage I.	Stage I.	0	4
Stage I.	Stage 0.	1	1
Total		14	16
Change in state		n (%)	n (%)
0		1 (8)	5 (31)
1		5 (36)	5 (31)
2		4 (28)	4 (25)
3		4 (28)	2 (13)
Total		14 (100)	16 (100)

Side-effects

Headache, sleep disorders, nasal congestion, fatigue, phlegm/catarrh in the back of the throat and cough are common symptoms of CRS, and thus they can't be considered side-effects of the therapy. Short-term nasal burning was reported by 6 patients in the AmB group, and only 1 patient mentioned dryness of the nasal mucus and bleeding in the placebo group. The therapy did not have to be interrupted because of side-effects in any of the cases.

Our evaluation of the preoperative and postoperative CT scans in the AmB group did not reveal an improvement in the scores; the data were scattered significantly. However, an improvement was noted in the placebo group. The comparison of the extents of change in the two groups did not indicate a significant difference ($p=0.052$). In this respect, mention should be made of the well-known clinical experience that the symptomatic recovery following

endoscopic sinus surgery does not correlate significantly with the extent of improvement seen on the CT scans (37, 38).

Our patients displayed marked improvements in the sinus complaints by the end of the 1-year course of treatment in both groups, though without a significant difference in the extent of change in the two groups. During the evaluation of the results, it should be borne in mind that AmB also exerts a direct toxic effect on the epithelial cells of nasal polyps, but does not damage the integrity of turbinate epithelial cells. Many authors explain the successful AmB treatment of nasal polyposis through this effect (39). It should be noted that no polyp tissue was present in our study population at the beginning of antifungal treatment which rules out any possible anti-polyp tissue effect of AmB. During the surgical procedures, the mucus in the sinuses, potentially containing fungal antigens, was thoroughly eliminated. These two facts may have contributed to the final outcome that the two groups did not exhibit a significant difference as concerns the changes in the symptoms even after 1 year. A significant difference was not observed in the quality of life test either; the explanation of this might be similar to that described in connection with the evaluation of the sinus complaints.

The assessment of the preoperative and the 12-month postoperative findings clearly shows that 1, 2 or 3-stage changes occurred more often in the AmB group, while an unchanged endoscopic finding was observed more frequently in the placebo group. However, in view of the relatively small number of patients, caution is advisable in the interpretation of this finding. Nonetheless, it is a fact that the endoscopic finding did not correlate with the changes observed in either the primary or the secondary outcome measures.

D. Evaluation of patient adherence and adverse events

1. Significance of compliance measurement

As mentioned before there are several factors that need to be taken into consideration when evaluating the outcome of different publications. Thus throughout the planning and the organizing phase of our study we were focusing on such factors which could have an influential effect. The knowledge of patient adherence (the extent to which the patient’s actual history of drug administration corresponds to the prescribed regimen) is important both in

medical research and in clinical practice. The results of clinical trials cannot be interpreted realistically without adherence information (40). The aim of our measurements was to assess patient adherence and to identify non-adherent patients during a 12-month double-blind clinical trial relating to the safety and efficacy of an AmB nasal spray self-administered for the treatment of CRSwNP. Because there is no gold standard for the measurement of adherence/compliance, the combination of two indirect methods (self-reporting and recording of the amount of medication administered) furnished better information concerning the attitudes of the patients and the factors influencing adherence. Assessments were made of how the adherence changed during the 12-month period, which factors influenced the attitudes of the patients and what adverse events were reported. The correlation between the adverse events and the adherence, and the comparability of the two adherence measurement methods were considered.

2. Methods

Participants

Thirty-three patients were recruited into the clinical study by the Department of Otorhinolaryngology, Head and Neck Surgery. Each patient was required to read and sign a form of informed consent and the study was approved by the Regional Research Ethic Committee of the University of Pécs Medical Centre. The patients ranged in age from 33 to 76 years (mean = 53,1 years); 50,0 % of them were male; the duration of their education ranged from 8 to 17 years (mean = 14,5); the number of scheduled medications in addition to the AmB nasal spray ranged from 0 to 8 (mean = 2,4). Thirty of the 33 patients completed the 12-month treatment period, they were requested to complete the Brief Medication Questionnaire (BMQ) and 27 did so at the last visit (response rate 90%).

Measures

Two indirect methods were used for the measurement of adherence: (a) recording of the amount of medication self-administered and (b) self-reporting by the patient via the Hungarian translated version of the standardized BMQ revised in 2003 (41). The amount of medication self-administered was recorded monthly by measuring the residual fluid in the spray containers to an accuracy of 0,1 ml (1 dose or unit = 0,1 ml). The cut-off point applied to define adherent patients was 80 %. Adherence was calculated according to the following:

$$Adherence\ (\%) = \frac{units\ issued - units\ returned}{units\ expected} \times 100$$

The accuracy of the BMQ was assessed earlier by using electronic monitoring with the Medication Event Monitoring System (Apres, Apria Healthcare, Fremont, California). The BMQ was translated into Hungarian by the authors and the wording of the instrument was edited for use with nasal sprays and adjusted to our study. Of the 16 questions in the BMQ three questions regarding the medication name, the reason for taking the medication and the amount paid were omitted since the sprays had codes, were the only medication involved in the study and were provided to the participating patients free of charge. Four questions were added to identify the use of other medications or nasal sprays and to ask how participants used the sprays throughout the whole 12-month trial period. The answers to these questions, not being in the original version, were not integrated into the Adherence Risk Scale (ARS) at the evaluation of the BMQ.

The *Regimen Screen* contained questions regarding the past week of treatment. The patients received a score of "1" if their answers to the questions indicated potential non-adherence and "0" if their report indicated adherence. The *Belief Screen* measured two beliefs that can be linked to non-adherence: doubts about the efficacy of the treatment, and unwanted side-effects or bothersome features. The patients received a score of "1" if they responded "not well" when asked about how the sprays work for them, and also if the sprays were defined as bothersome or unwanted side-effects were mentioned. The *Recall Screen* measured potential problems in remembering all doses. The question about multiple doses in the original BMQ was changed, since the patients in our study were instructed to use the sprays twice a day. Instead, two potential problems were included. The patients received a score of "1" if they answered "yes" to any of the questions. The *Access Screen* analyzed barriers. The

question about problems paying for the medication was excluded from our BMQ. Patients received a score of "1" if they answered "yes" to any of the three questions (see questions in *Appendix C.*).

During the evaluation of the BMQ the subtotals for each screen were calculated by adding up the scores for each question. If the score of a screen was ≥1, the subtotal for the screen was "1", and in that case the screen indicated potential non-adherence or the presence of the defined barrier. The ARS is a summary scale constructed by adding up the subtotals for each screen. It measures potential non-adherence and the number of factors influencing patient behaviour.

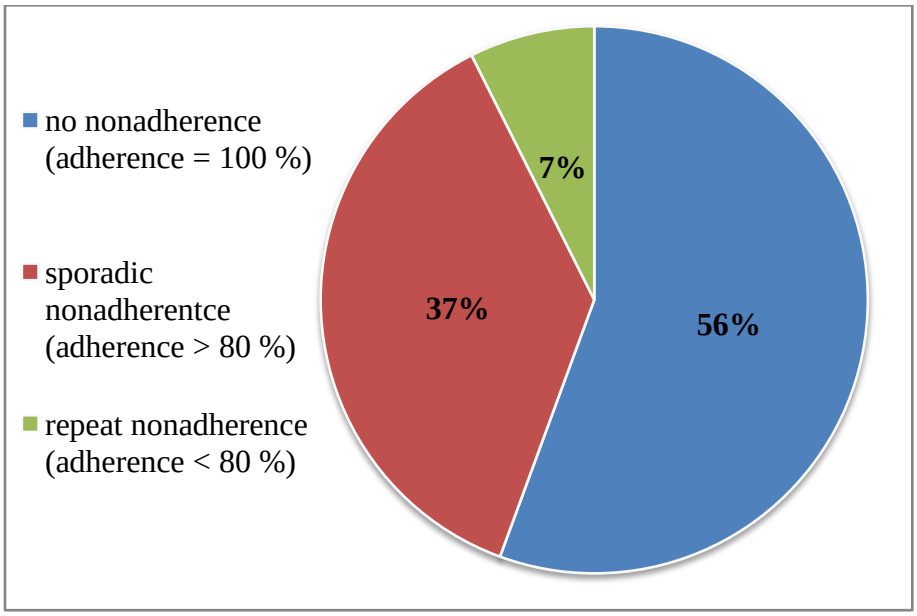
3. Results

The 12-month adherence to the Amphotericin B nasal spray

The records related to the amount of medication self-administered did not reveal any significant change in patient adherence between the first (97,5 ± 8,1 %) and the last (94,7 ± 11,5 %) month of the treatment (one-tailed paired sample t-test, p = 0,126). These findings correlate with the observation that patients appear to discontinue their medication or deviate from the recommended dosing at higher rates in practice settings than in randomized clinical trials (42). Even though a moderate decline was seen from month to month, at the end of the study the overall adherence was notably above the most commonly documented cut-off point for adherence of 80 %. The difference in compliance between the AmB (97,3 ± 8,1 %) and placebo (97,2 ± 7,4 %) groups (two-tailed independent sample t-test, p = 0,323), or between the male (97,8 ± 7,4 %) and female (96,6 ± 8,1 %) patients (two-tailed independent sample t-test, p = 0,886) during the 12 months was not significant

Figure 7. shows the compliance of patients with drug therapy during the 12th month of the study determined from the recorded amount of medication self-administered. Approximately half of the patients used the prescribed amount of spray, 37 % missed not more than 20 % of the doses, and only 7,4 % were considered to be non-adherent. Interestingly the 2 patients who had the lowest compliance rates through the whole study period were closely related and lived in the same household.

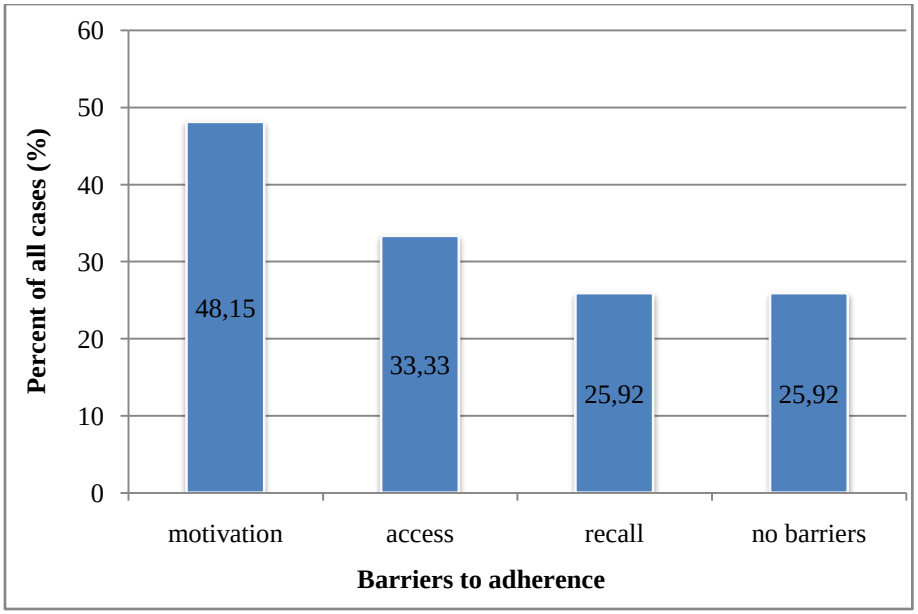
Figure 7. Patient adherence according to the recorded amount of medication self-administered during the 12th month of amphotericin B nasal spray treatment.



Patient adherence determined by self-reporting in the BMQ

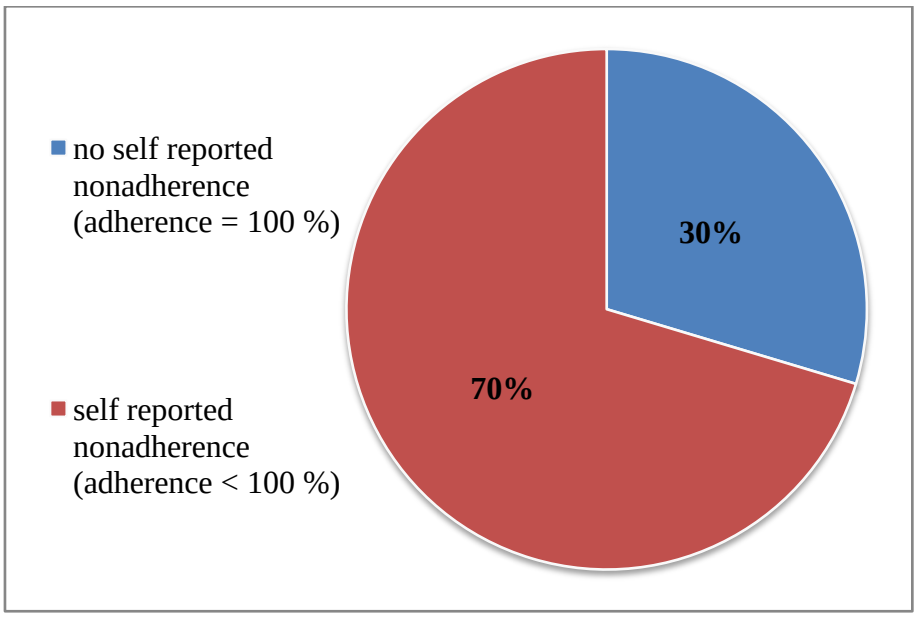
Through evaluation of the scores obtained in the BMQ, the ARS can be constructed. This tool not only indicates non-adherent behaviour, but also provides information regarding the factors influencing adherence. The BMQ defines very strict conditions. By admitting a missed dose a patient is classified as potentially non-adherent. This might result in the previously determined lower sensitivity (80 %) and high specificity (100 %) of the instrument (41). Accordingly it is likely that the BMQ underestimates adherence and overestimates non-adherence.

Figure 8. Adherence Risk Scale results demonstrating barriers to adherence



Among those patients whose recorded data did not indicate non-adherence, in two-thirds of the cases the BMQ demonstrated at least one barrier to adherence. The most common barrier to adherence was motivational, since 48,1 % of the patients mentioned negative beliefs concerning the efficacy of the treatment or bothersome side effects. An access barrier was identified for every third patient, most mentioning a malfunction of the spray pump or running out of medication too early. Only 25,9 % of the patients admitted or implied the existence of recall barriers (**Figures 8 and 9.**).

Figure 9. Adherence Risk Scale results demonstrating self-reported non-adherence



The addition of four questions to the BMQ regarding the regularity of use throughout the whole study period yielded more detailed information. The following results were obtained. Intra-year regularity: 85,2 % answered “constant regularity”, 11,0 % of the patients used the sprays “more regularly during the early months” and only 3,7 % admitted “irregular use”. The regularity of missed doses: 44,4 % “never missed a dose”, 44,4 % “missed a dose around once a month”, 3,7 % missed doses occasionally (1-2 doses a week), and only 2 patients (7,4 %) missed doses every day. When use of the sprays was compared between the two groups, the adherence in the placebo group always proved to be higher. Fewer doses were omitted and the regularity of use was also higher. This observation may be linked to the frequency of adverse events experienced.

Adverse events

AmB applied topically may produce local irritation, pruritus, and skin rash (43). The previously discussed seven clinical studies documented various side-effects in connection with local AmB treatment such as nasal mucosal inconvenience and a burning sensation, nausea or headache. The incidence of adverse events amongst patients treated with AmB nasal sprays or lavages in those studies lay in the range from 13,3-66,0 %.

During our study the following side-effects were reported in the AmB group: local irritation (7,7 %) and headache (7,7 %); while in the placebo group: loss of smell (7,1 %) and local irritation (7,1 %) was mentioned. It should be noted that loss of smell and local irritation can also considered being symptoms of CRS. The regularity of the observed adverse events is summarized in **Table 5.**

Table 5. Adverse events in the two groups of patients receiving amphotericin B nasal spray or placebo. (Data from 27 patients who completed the BMQ.)

Incidence of adverse events	Amphotericin B		Placebo	
	N	%	N	%
Once	0	0	1	7,1
Sometimes (once a month)	4	30,7	2	14,2
Regularly (once a week)	0	0	1	7,1
Always	3	23	0	0
Total	7	53,8	4	28,6
Never	6	46,2	10	71,4

According to our findings the incidence and also the regularity of side-effects were higher in the AmB group than in the placebo group. The overall incidence in the AmB group was 53,9 %, as compared with only 28,6 % in the placebo group.

Comparison of the adherence measurement methods

The combination of the two methods is considered superior to reliance on either of the single methods, since both have advantages and disadvantages. Recording of the amount of medication self-administered gives a more precise and objective continuous variable, but no details about the circumstances of drug use or factors influencing the attitude of the patients can be measured. Such recording of drug use have been found to overestimate the actual adherence behaviour (40). This holds for our study too, since 92,6 % of the patients were defined as adherent (adherence > 80 %) on the basis of the medication records. The scoring of the BMQ is too strict and its sensitivity is lower than its specificity; thus it underestimates the actual adherence. Consequently, we considered it to be less reliable for the numerical determination of patient adherence. We had to find that only 29,6% of our patients were categorized as totally adherent (adherence = 100 %) by the Regimen Screen. Hence, no statistical correlation exists between the outcomes of the two methods (Fisher's exact test: χ^2 = 4,29; df = 1; p = 0,87). The actual adherence is most likely somewhere between the two adherence results. Another problem we had to face was that the spray-holders contained only the amount of solution necessary for a 30-day period. If the patient used the spray incorrectly,

for example administering too many doses at a given time, the omission of doses on different occasions could not be measured with this method. Additionally, improper handling or storage of the spray-containers may have resulted in leakage and the loss of spray solution, thereby resulting in falsely high adherence measurement results.

It can be stated that patient adherence was relatively good; only a moderate decline was seen during the 12 months. At the end of the study, the overall adherence was 94,7 ± 11,5 %. During the last month of treatment, 55,6 % of the patients were totally adherent, in 37 % sporadic non-adherence was measured, and only 2 patients (7,4 %) were categorized as non-adherent. Such a high adherence rate might be explained by the fact that most of the present patients suffered from symptoms which greatly deteriorate the quality of life, and had had several previous operations; accordingly, they were very determined and had a good awareness of their medical state. Moreover, during the study a good relationship was created between the patients, the physician and the pharmacist. The ARS constructed from the scores of the BMQ indicated the presence of self-reported non-adherence and factors influencing adherence. From the ARS, we concluded that motivational barriers influenced the adherence in 48,1 % of the patients. Some patients had doubts about the efficacy of the treatment, and most of the patients (especially in the AmB group) experienced side-effects or bothersome features. Access barriers occurred in 33,3 %, probably because of an inappropriate use or dosage of the medication or occasional malfunctioning of the spray apparatus. Recall was the least frequent (25,9 %) barrier to adherence.

Although the drug intake records did not indicate a significant difference in adherence between the two genders or the two medication groups, the answers to the BMQ revealed that patients deviated from the prescribed dosing regimen more often in the AmB group. This observation might be linked to the higher incidence of side-effects caused by AmB.

E. Conclusions and overview about amphotericin B antifungal treatment in Chronic Rhinosinusitis with Nasal Polyposis

Fungal organisms can give rise to extremely serious acute and chronic sinonasal infections in immune-compromised hosts (8, 11, 12). The fungal etiology of CRS in immune-competent patients is still a topic of considerable debate and needs further clarification (8, 12, 17, 20, 44, 45). AFRS is a well-known, but relatively rare form of CRS, which is characterized by IgE-type immune reactions against fungi cultured from the sinuses. Collins et al. (46) reported the presence of local specific IgE to fungi in the EM of some CRS patients, but even in those cases no systemic IgE to fungi was detected. They also noted the presence of local specific IgE to fungi in all the CRS patients, with positive fungal cultures. The consensus of the Workshop on Fungal Sinusitis on terminology and disease classification of fungal rhinosinusitis has been published recently (9).

However, there are at least three reasons why some investigators doubt the relevance of fungi in CRS. First, the incidence of fungal colonization in patients with CRS is similar to that in healthy individuals (3, 17, 20). Secondly, the presence of EM is not always associated with a positive fungal culture, and moreover the group of CRS patients with EM includes subgroups based on the presence of fungi in the EM and on IgE-mediated fungal hypersensitivity that share clinical characteristics (16). The third important fact is that bacteria have also been isolated in the sinus cavities of patients with positive fungal cultures; it therefore remains unclear whether intranasal fungal antigens exacerbate the chronic inflammatory response or are simply present coincidentally in patients with CRS (3).

There are important clinical observations too which cannot be ignored and which point to the possible etiological role of fungi in CRS. These are as follows:

1. Gosepath et al. (47) recently successfully proved the presence of DNA fragments of *Alternaria* species in the sinonasal mucous membranes of patients with CRS, concluding that the fungal antigen is transported from the EM to the small vessels of the nasal mucous membranes by the antigen presenting cells (macrophages), triggering eosinophil infiltration.

2. Shin (45) demonstrated that the levels of serum IgG to *Alternaria sp.* and *Cladosporium sp.* were higher in patients with CRS than in healthy individuals.

3. The presence of specific IgG antibodies correlated with increased IL-5 and IL-13 levels during exposure to *Alternaria sp.* in vitro, but this did not hold controls (45).

4. Ponikau et al. (18) reported that treatment for 6 months, with regular application of a nasal lavage containing AmB resulted in decreased concentrations of both neurotoxin and IL-5 originating from eosinophilic cells. In contrast, the concentrations of both mediators increased in the placebo group, though the changes were not significant. The observations of other researchers on the inflammatory mediators did not confirm these results (48, 49). If the fungal etiology is true, it would be reasonable to recommend antifungal treatment to patients with CRS in order to decrease or eliminate the fungal load and achieve a symptomatic improvement.

The question arises as to what other causes could have led to the ineffectiveness of AmB treatment in almost half of the previously published (methodologically not always correct) clinical studies (**Table 1.**). There are a number of possibilities:

1. CRS is a multifactorial disease: immune deficiency, genetic factors, anatomic variations, atopy and environmental factors such as air pollution and smoke are predisposing factors (3).

2. The compliance may not have been satisfactory during the course of long-lasting examination protocols and it was usually not taken into consideration in earlier clinical studies (31).

3. Differences in sensitivity of the various fungi to AmB (50).

4. Differences in effectiveness of the mechanisms repairing the direct membrane damage and the consequences of oxidative stress (51).

5. In patients with advanced CRSwNP the amount of drug that penetrates to the bottom of sinuses filled with EM and polyps may be insufficient.

The experience gained so far indicates that the results of oral antifungal treatment are rather confusing (52, 53). Our clinical study does not justify the need for the administration of AmB nasal spray for 12 months following endoscopic nasal polypectomy. The methodological errors involved in the studies conducted so far lead us to think that it is an oversimplified explanation that fungi are “innocent bystanders”. Our results and the data

available draw attention to the necessity of integrated further clinical studies, with improved methodology. Treatment modalities should be applied on a case-by-case basis. Future research should focus on the selection of the CRS patients who will certainly benefit from antifungal therapy (15, 16). Particular attention should be paid to the diagnosis and treatment of patients with aspirin intolerance among those who require revision surgery, and to the recognition of those patients who will benefit from specific immunotherapy or prolonged low-dose macrolide therapy (3).

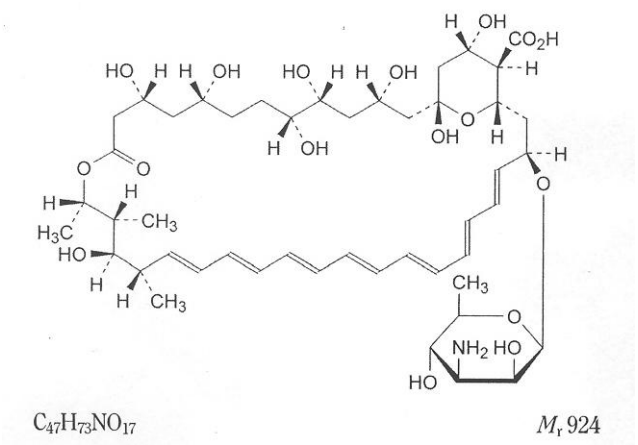
V. Section Two: Analytical part

A. Introduction of polyene antibiotics and the possibilities for analytical methods

AmB is a polyene antifungal agent produced by *Streptomyces nodosus.* Like other polyenes (for example nystatin, natamycin or meparticin) AmB has a cyclic structure closed by an internal ester bond and consists of two parts: (a) a rigid apolar part of conjugated double bonds (seven in the case of AmB) which is responsible for the marked lipophilicity of the molecule; and (b) a polar part comprising a large number of hydroxyl groups. It also possesses an amino sugar (mycosamin) and a carboxyl group that is present as zwitterions. These properties of AmB are responsible for the comparative insolubility of the compound in organic solvents (**Figure 10.**). Although it is a particularly insoluble substance in inorganic solvents as well, it can be dissolved in DMSO and dimethylformamid. The presence of bile salts (sodium deoxycholate) allows the formation of mixed micelles with AmB and solubilisation of the antibiotic. Its antifungal spectrum is very broad and is not restricted to fungi since polyenes are active against certain protozoa and algae, but do not possess any antibacterial or antiviral activity. AmB is the major first-line antifungal agent and is considered to be the most effective in a number of clinical systemic mycoses, however its use can be limited by its toxicity when used parenterally, whereas orally its tolerability is excellent since it is very poorly absorbed (<5%). The very great majority of fungi are susceptible to very low concentrations of AmB, generally at a MIC of <0,5 μg/ml. In vitro AmB exerts fungicidal activity after 3 hours, but this activity does not persist for more than 48 hours (26, 54).

AmB's antifungal effect (fungicidal or fungistatic, depending on the applied concentration) is based on the interaction of AmB with plasma membrane sterols and other AmB molecules, in which it forms pores. As hollow cylinders are formed within the lipid bilayer the ions and the essential cell constituents can escape, resulting in the death of the cell. The AmB-sterol complex induces disorganization of plasma membrane by this way decriptification of cell constituents. The selectivity of AmB for the fungal cell originates from the greater affinity for ergosterol – the major component of fugal membrane-, than for cholesterol present in mammalian membranes (26).

Figure 10. Chemical structure of amphotericin B (formula from Ph. Eur. 6.)



Analysis and quantitative determination of polyenes can be carried out with different methods such as chemical and microbiological analysis. The chemical measurements are based on the detection of the light absorption of the unsaturated chromophore segment of the molecule. Samples containing AmB can be measured directly with spectrophotometry or after chromatographic separation. During the past two decades several HPLC methods have been published and used (55, 56). With the aid of microbiologic detection methods directly the antimicrobial effect can be measured. Such methodologies are agar diffusion and turbidimetric bioassays (54, 57). Bioassay measurements or methods were rarely published even though microbiological assays are the official methods for the quantitative analysis for AmB (and also nystatin) prescribed by the American (USP. 21.) and European (Ph. Eur. 6.) pharmacopoeias. Bioassays are favoured because of the chemical instability of the molecule and the homogeneity and purity of the preparations might differ due to the fermentation and extraction procedures.

The two types of analytical methods differ fundamentally from each other. With the aid of chemical methods, those parameters (polarity, light absorption) are studied which are directly associated to the molecular structure of the molecule. The technical, financial and instrumental requirements of such measurements are higher - for instance HPLC apparatus is required -, but they are relatively fast and reproducible quantitative measurements can be performed. From the aspect of the measurement of biologically active substances, a major disadvantage is that no information can be gathered regarding the antimicrobial effect of the examined samples. On the other hand with the aid of biological assays directly the biological

effect can be measured. It should be emphasized, that such methods are typically more time consuming and the reproducibility is not as good with chemical analysis.

B. The question of stability and optimal storage conditions for clinical study medications

As emphasized earlier in the clinical part of the thesis, the stability testing of study medications is an important factor because, on one hand patients needed to use the sprays for a year long period and on the other hand the long term stability of AmB is questionable in water based solutions.

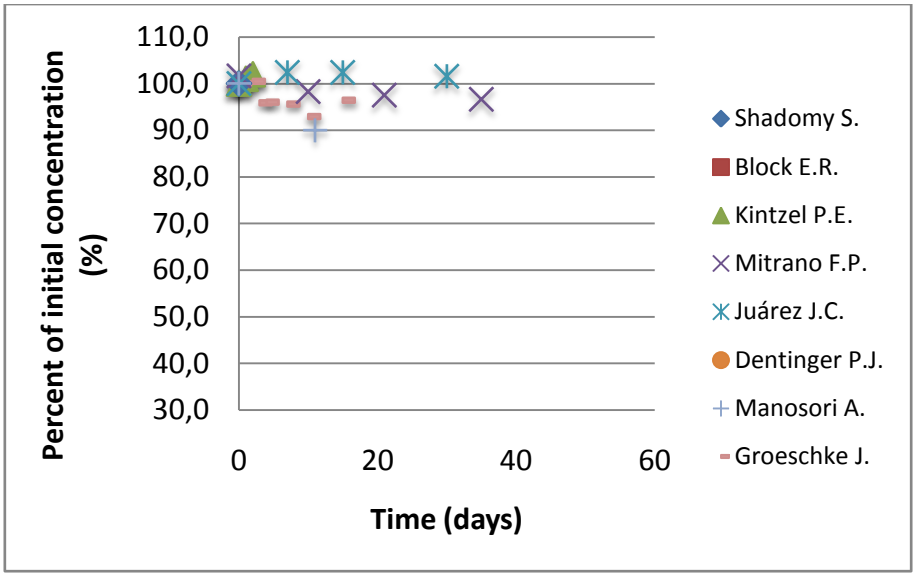
Previously published papers were screened and reviewed to evaluate the question of stability. Our results are summarized in **Table 6.**. Also the outcomes of these studies are illustrated in **Figures 11. and 12.** from the aspect of storage temperature.

It can be seen in the table, that earlier microbiological assays were used for the analysis, but during the last two decades chromatographic determinations were applied. From this aspect the comparison of the results could be rather difficult, but since these studies are short term (less than 24 hours), this problem is of no significance. The tested solutions are of different AmB concentrations ranging from 0,05-100 mg/ml and were all made of commercially available pharmaceutical preparations. Five percent dextrose, as the only recommended solvent of dilution for Fungizone, was used most commonly. For the sake of easier comparability and transparency only the results of light protected samples are presented. It has to be stated, that not all authors have tested the effect of light exposure. When comparing the results of studies using different containers for the solutions, the conclusion can be drawn, that probably no strong correlation exists between the material of the container and the stability.

Table 6. Summary of published stability tests of amphotericin B solutions.

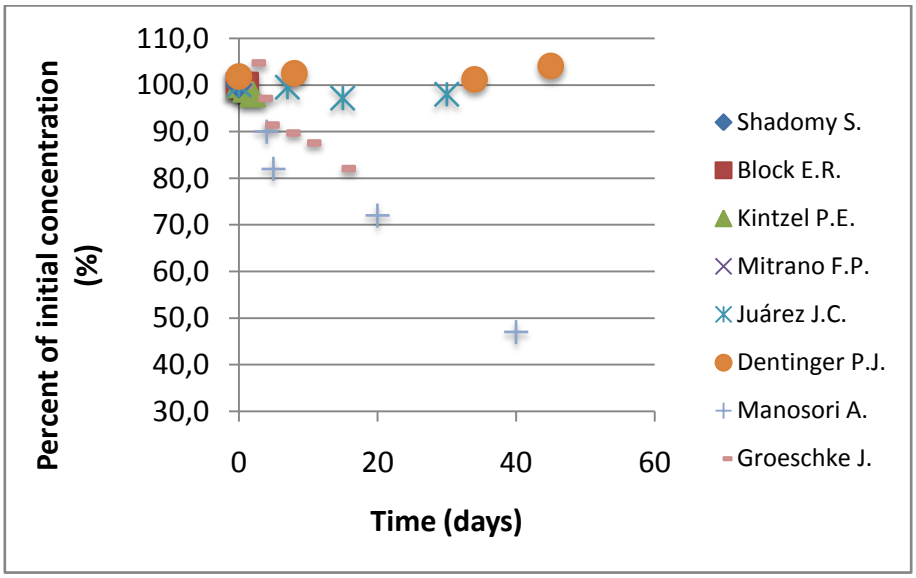
Author	Shadomy S. (58)	Block E.R. (59)	Kintzel P.E. (60)	Mitrano F.P. (61)	Juarez J.C. (62)	Dentinger P. (63)	Manosori A. (64)	Groeschke J. (65)
Year	1973	1973	1991	1991	1997	2001	2003	2006
Method	Bioassay	Bioassay	HPLC	HPLC	HPLC	HPLC	HPLC	HPLC
Concentration	0,05 mg/ml	14 mg/ml	1,4 mg/ml	0,25 mg/ml	1 mg/ml	100 mg/ml	5 mg/ml	7,4 mg/ml
Prepared from	Fungizone	-	Fungizone	Fungizone	Fungizona	Fungizone	Fungizone	Fungizone
Excipients	5 % dextrose, 0,2 % NaCl	-	5 % dextrose	5 % dextrose	-	citric acid, sodium phosphate	-	1,4 % sodium hydrogen carbonate
Container	Glass	Glass	PAB	PVC	Glass	Polyethylene	Glass	Glass
Protected from light	yes	yes	yes	yes	yes	yes	yes	yes
Stability (4-6 °C)	≥8 hours	-	≥1,5 days	≥35days	≥30 days	-	11,4 days	≥15 days
Stability (20-25°C)	≥8 hours	≥1 day	≥1,5 days	-	≥30 days	≥93 days	4,1 days	4 days

Figure 11. Stability of amphotericin B solutions stored below room temperature (4-8 °C)



On Figure 11. and 12. data from Table 6. are plotted. It can be seen that at lower temperature (4-8°C) the solutions are more stable. The refrigerated samples are considered to be relatively stable and the outcomes of the studies are in correlation with each other. This statement not holds for samples stored at room temperature. The longest stability test designed by Dentinger et al. shows no loss of concentration at 20 °C for more than 30 days, while Manosori and Groeschke concludes, that samples are stable for not more than 4 days at room temperature.

Figure 12. Stability of amphotericin B solutions stored at room temperature (20-25 °C)



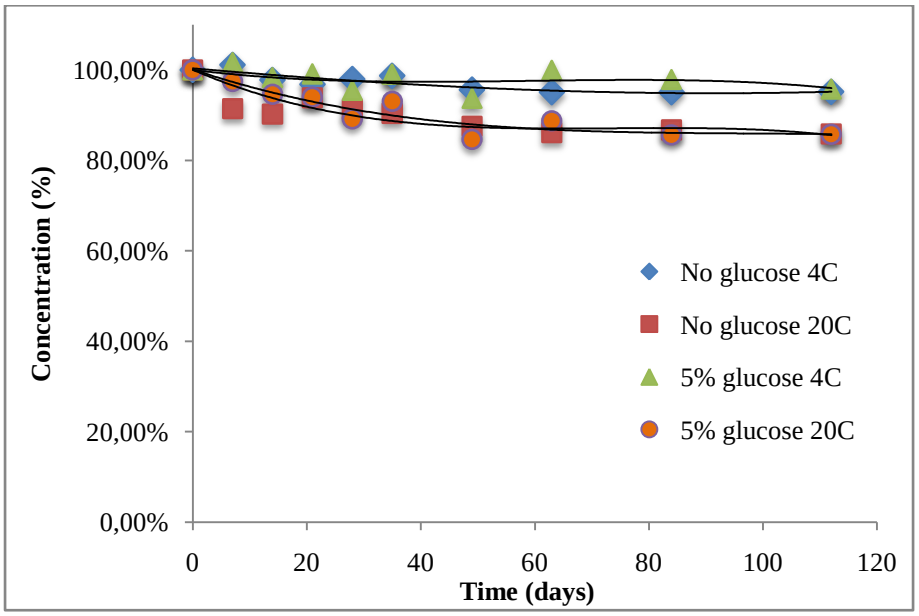
The correct interpretation of these controversial results is rather difficult and could be misleading, so when the question of stability arose during the preparation of the pilot clinical study, the organization of a new stability test seemed to be the most reliable source of information. As mentioned before, bioassay if the official quantitative analytical method for polyenes, thus the combination of both chemical and microbiological methods was proposed since this way more detailed information could be gathered regarding the physic-chemical changes in the samples.

C. Results of the stability test performed with spectrophotometry and agar diffusion. Contrast between the outcomes of the analytical methods.

In our study we investigated the stability of 5mg/ml AmB solutions (Fungizone®) with chemical (spectrophotometry) and biological (bioassay) detection. The effect of storage temperature and the addition of 5% glucose were evaluated on the stability of the solutions for three months.

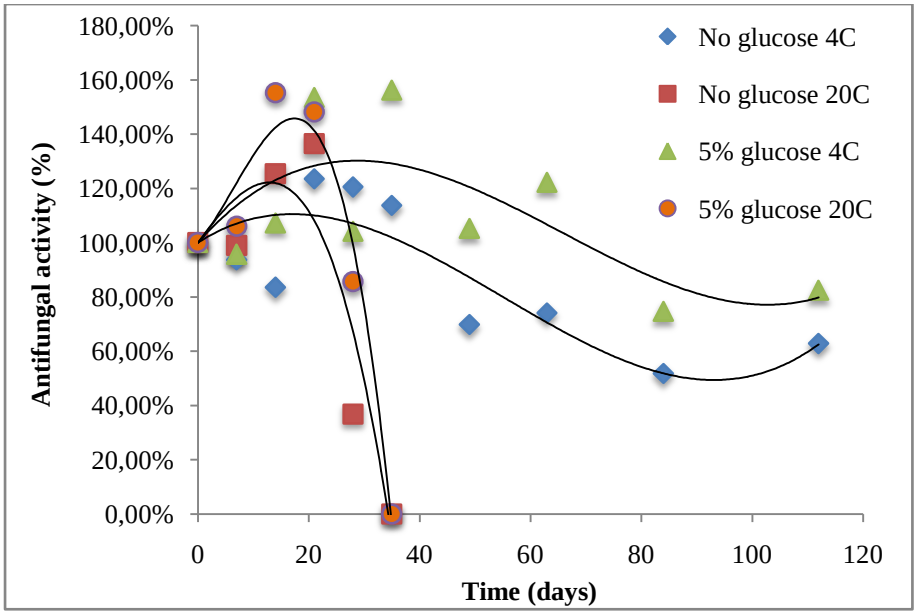
Study samples were prepared from Fungizone® (Bristol-Myers Sqiubb) which was diluted in 10,0 ml water for injection to give 5 mg/ml solutions. For the 5 % glucose containing samples glucose solutions (from glucosum anhydricum, Ph.Eur.5, Hungaropharma) were sterilized with 0,22 μm membrane filter. During spectrophotometric quantification 0,1 N NaOH was used for dilution and *Candida albicans* ATCC 90028 test strain was applied for bioassay. The composition of the test agar medium was: LAB-LEMCO (Oxoid) 5,0g; peptic peptone (Reanal) 10,0g; NaCl (Ph.Eur.5., Hungaropharma) 5,0g; Na_2HPO_4 (Reanal) 4,0g; Agar-agar (Merck) 20,0g; and 1000 ml deionised water.

Figure 13. Results of the spectrophotometric measurements show only minor changes in concentration.



The 3,33 ml stability samples (n=3) were stored in refrigerator (4 °C) and at room temperature (20-23˚C) in light protective glass containers. During the 112 day period, on 0., 7., 14., 21., 28., 35., 49., 63., 84. and 112. days, a 20 µl sample was removed from the container and used for the two analytical procedures. Chemical determinations were performed with Specord M40 spectrophotometer at the absorption maxima of AmB (265, 384 and 407 nm). Results are displayed as the averages of the three measurements for each sample. Microbiologic measurements were done in 18 cm diameter glass petri dishes filled with 120 ml assay medium inoculated with the test organism. Holes with the diameter of eight millimetres we cut in the agar medium and 200 µl of calibration or study samples were applied in them. Freshly prepared Fungizone was used for the production of calibration solutions and sterile deionised water was used for the dilution of these samples. The petri dishes were incubated at 30 °C for 24 hours and the inhibition zones were measured with a 0,1 mm precision scale. The dose response curve was determined and with the above mentioned conditions it proved to be linear in the concentration range of 0,625-5,0 µg/ml ($r^2 \approx 0.96$). From the diameter of the inhibition zones, the concentrations of the samples were calculated.

Figure 14. Bioassay measurements indicate major changes in antifungal activity.



The two detection methods showed strikingly different results. According to the chemical analysis the samples are considered relatively stable under all observed conditions (loss of concentration is: 14,2% at 20°C and 4,5% at 4°C) as can be seen on **Figure 13**. As opposed to chemical analysis, bioassay (**Figure 14.)** shows complete loss of antifungal activity after 35 days of storage, at room temperature and notable decrease can be observed at 4°C (glucose containing solution 17,6%; glucose free solution 37,2%). Storage temperature had significant effect ($p<0,05$), while 5% glucose had no significant effect ($p>0,05$) on the stability of the examined solutions. The results of our measurements with quantitative data (mean ± SD) are summarized in **Table 7**. We estimated the shelf life of the glucose-free solutions, being stored at 4°C, to be 30 days in accordance with our bioassay results. Interestingly in the case of bioassay, during the first half of the test period the antifungal activity did not decrease, but instead seemed to increase under all examined circumstances. For the samples stored at room temperature this positive change in activity is even more intense, also the presence of glucose seemed to have an additive effect in this phenomena.

This information drew our attention to the major differences that can be detected with the two different methods. There are presumptions, but no facts that could explain our observations. Most probable is that colloidal change occurs in the solution resulting in the decrease in capability of AmB to diffuse within the agar layer, this way producing smaller inhibition zones. Unfortunately this theory does not answer the initial increase in antifungal

activity. It has been reported by Romanini et al. (66) that AmB in aqueous medium forms higher molecular weight aggregates which decreases it's activity. The size of these is estimated at 200 molecules. It has also been published, that AmB/sterol channels are produced in the fungal membrane, consisting of eight AmB and eight sterol molecules (67). It could be possible, that during the initial self association phase, when smaller aggregates of just couple of molecules exist, these channels might form faster or more efficiently, resulting transitionally in a more potent microbiological activity.

Table 7. Stability test results for 5mg/ml amphotericin B solutions (Fungizone®) with chemical (spectrophotometry) and biological (bioassay) detection. (mean±SD, n=3)

Spectrophotometric measurements						**Bioassay measurements**					
Time	Temp.	glucose-free samples		glucose containing samples		Time	Temp.	glucose-free samples		glucose containing samples	
		mean	SD	mean	SD			mean	SD	mean	SD
0. day	20 ºC	100,0	± 0,37%	100,0	±0,37%	0. nap	20 ºC	100,0	±5,06%	100,0	± 9,83%
7. day	20 ºC	91,4	± 0,27%	97,5	±4,43%	7. day	20 ºC	99,0	±23,88%	106,1	±25,92%
14. day	20 ºC	90,2	± 1,11%	94,6	±0,57%	14. day	20 ºC	125,4	±13,72%	155,2	±27,71%
21. day	20 ºC	93,9	± 1,33%	93,9	±1,31%	21. day	20 ºC	136,5	±18,05%	148,2	±13,92%
28. day	20 ºC	91,3	± 1,29%	89,2	±1,21%	28. day	20 ºC	36,8	±6,22%	85,6	±8,36%
35. day	20 ºC	90,4	± 1,23%	93,0	±1,94%	35. day	20 ºC	0,0		0,0	
49. day	20 ºC	87,5	± 1,30%	84,6	±1,12%	49. day	20 ºC	0,0		0,0	
63. day	20 ºC	86,1	± 1,50%	88,7	±1,40%	63. day	20 ºC	0,0		0,0	
84. day	20 ºC	86,7	± 0,65%	85,7	±0,76%	84. day	20 ºC	0,0		0,0	
112.day	20 ºC	85,9	± 1,01%	85,8	±0,39%	112.day	20 ºC	0,0		0,0	
0. day	4 ºC	100,0	± 1,07%	100,0	±1,07%	0. day	4 ºC	101,5	±5,06%	100,0	±9,83%
7. day	4 ºC	101,1	± 1,85%	101,6	±0,97%	7. day	4 ºC	93,6	±45,43%	95,8	±21,62%
14. day	4 ºC	97,8	±1,11%	98,3	±0,53%	14. day	4 ºC	83,6	±1,62%	107,2	±25,04%
21. day	4 ºC	96,9	± 017%	99,1	±1,07%	21. day	4 ºC	123,5	±20,55%	153,5	±7,20%
28. day	4 ºC	98,0	± 1,44%	95,4	±0,96%	28. day	4 ºC	120,5	±28,76%	104,1	±22,27%
35. day	4 ºC	98,7	± 4,32%	99,1	±0,50%	35. day	4 ºC	113,7	±23,07%	156,2	±7,25%
49. day	4 ºC	95,6	± 0,64%	93,7	±0,20%	49. day	4 ºC	69,8	±16,62%	105,2	±16,07%
63. day	4 ºC	95,1	± 1,74%	99,9	±0,57%	63. day	4 ºC	74,0	±14,41%	122,2	±4,54%
84. day	4 ºC	95,1	± 0,34%	97,9	±1,08%	84. day	4 ºC	51,8	±9,01%	74,7	±20,33%
112.day	4 ºC	95,2	± 0,24%	95,8	±0,95%	112.day	4 ºC	62,8	±12,21%	82,4	±27,91%

Nevertheless it can be stated, that such changes occur in the solutions that do significantly affect the microbiologic effectiveness of AmB while the chemical structure (principally the polyene segment and its light absorption properties) is not altered significantly. Thus the proper investigation of stability is of great importance when determining the shelf life of medications. After the stability test had been completed, our intention was to do the optimization of chemical and biological analytical methods in the interest of getting better understanding of the changes occurring in the solutions. The

chemical analysis can be improved if a more sensitive and detailed test method is used, such as high-performance liquid chromatography (HPLC). With the aid of chromatographic separation not only the identification and quantification of AmB can be achieved, but also the separation of degradation products is possible. In addition, a more precise bioassay method was needed to reduce the coefficient of variation when measuring antifungal activity. In this way the initial activity increase and the following decrease can more reliably be documented and its importance can be evaluated.

D. Chemical methods

1. Introduction

As described previously, chemical methods are suitable for the identification and quantification of the antifungal substance. By the application of chromatographic methods, not only the amount of polyene molecules (containing the conjugated double-bond segments) can be determined, but also separation allows us to distinguish molecules with different physicochemical properties.

Main, minor components and degradation products were screened for in Fungizone (Bristol-Myers Squibb). Chromatographic parameters, solvent composition and pH were altered to identify best parameters for optimal separation with HPLC. We intended to optimize earlier methods to achieve the best available separation of heptaene and tetraene components and to work out a method for quantification of the main component.

With TLC, the separation and visualization of the minor heptaene and tetraene components is more difficult to achieve due to the small concentration of the substances (approximately 1 per cent of the main heptaene AmB). Even so we intended to separate the main component from the others so that by the application of microbiologic detection (the method of direct bioautography is described in *Chapter VI.E.3.* of my thesis) antifungal activity could be visualized of the minor components or the degradation products. In this manner our main goal was to achieve reliable and reproducible separation for AmB with eluents suitable for biologic detection

2. HPLC measurements

a) Materials and methods

Our method was adapted and further improved based on previously published and evaluated literature data presented in **Table 6.** The HPLC system consisted of: Waters 600 pump, Rheodyne 7125 injector, Waters 996 PDA detector and Millenium software. Measurements were performed at ambient temperature. A volume of 25 µl samples were injected onto 250 x 4,6 mm C_{18} (Chromsil C18, 6 µm) columns, with a flow rate of 0,25 ml/minute. Heptaene components were measured at 407 nm, while tetraenes were determined at 305 nm. HPLC grade solvents were used for the eluents: acetonitrile (MeCN, Chromasolv, Sigma-Aldrich), methanol (MeOH), sodium acetate - EDTA buffers (4,1 g NaAc + 1,1 g Na_2EDTA +1000 ml deionised water) set to different pH values with acetic acid. Nystatin (Hungaropharma, Ph.Eur.5.), piroxicam (Hotemin inj., EGIS) and 4-nitro-1-naphthylamin (Aldrich) were tested as potential internal standards.

b) Results and conclusions

Although earlier publications did not lay emphasis on, or did not document presence minor components in AmB samples, even after preliminary measurements we could visualize several minor components in the chromatograms. To improve separation, the optimization of solvent system was performed and the following observations were made: increasing the ratio of buffer (**Figure 15.a.**) or augmenting the percentage of methanol within the organic phase (**Figure 15.b.**) improves the separation of different minor components, but at the same time notably increased retention times.

Figure 15.a. Effect of the ratio of buffer in the eluent system on separation

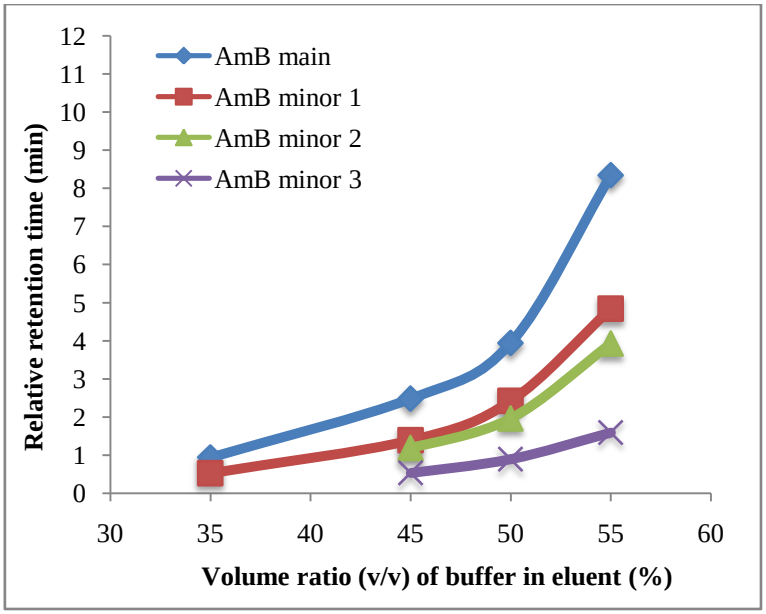


Figure 15.b. Effect of the ratio of methanol within the organic phase on separation

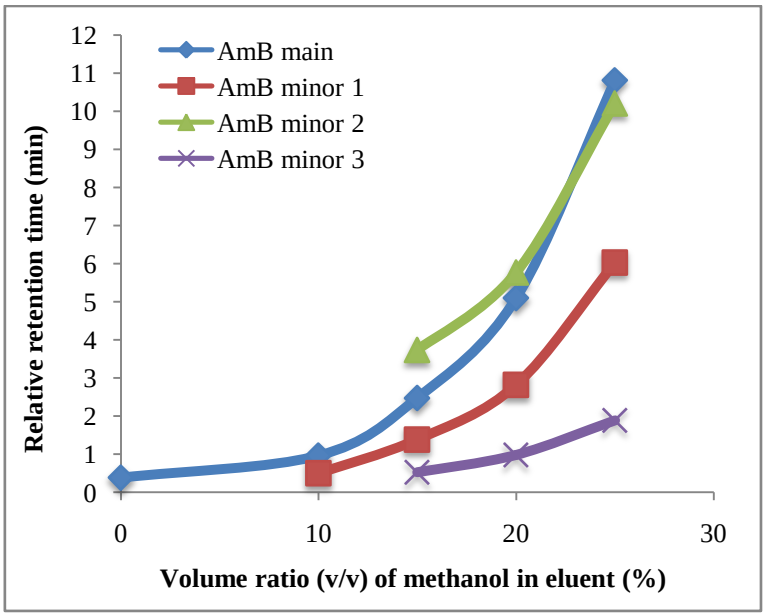
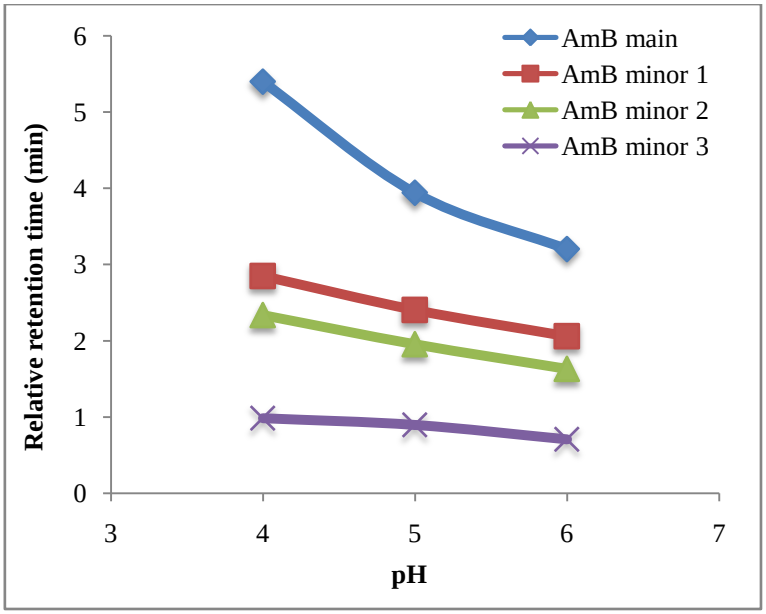


Figure 15.c. Effect of buffer pH on separation



When lowering the pH of the buffer (**Figure 15.c.)**, the separation of minor components from the main heptaene AmB is better. Unfortunately most tetraenes (amongst which amphotericin A can be found) are in such small proportion, that documentation and identification is rather difficult and cannot be well separated from heptaenes found in higher concentrations. With our optimized eluent system and the aid of the gradient program described in **Table 8.**, four heptaene components can be detected besides AmB main component at 407 nm. The amount of these constituent is about one hundredth of the main AmB. Only traces of tetraenes can be detected in Fungizone at the wavelength of 305 nm (**Figure 16.**). Probably amphotericin A - the most important minor component of AmB, which does not have antifungal effect - is amongst these peaks.

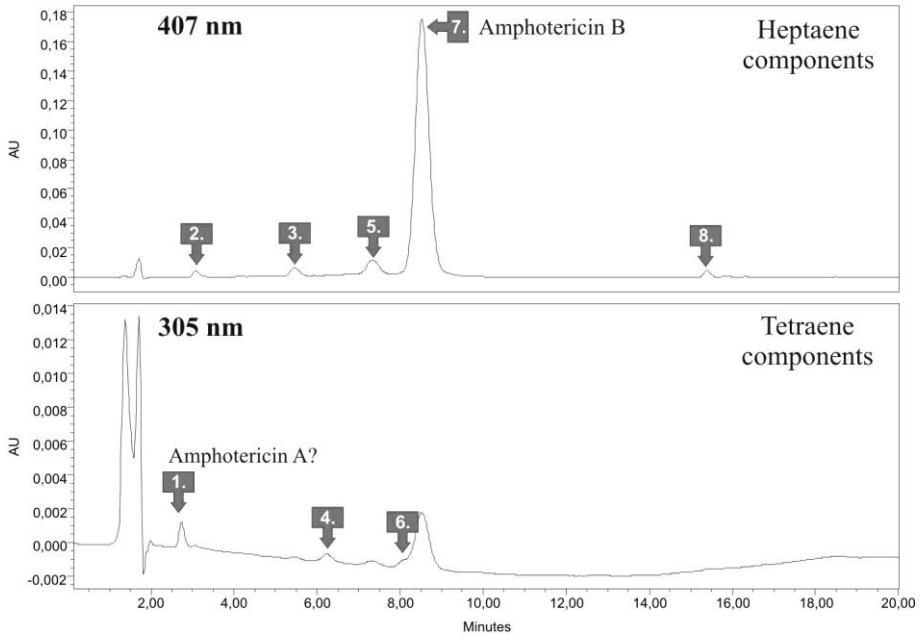
From the previously used internal standards we have tested nystatin, piroxicam and 4-nitro-1-naphthylamint and had to find that the later proved to be the best, because nystatin's spectrum and retention overlapped with the tetraenes and piroxicam's absorption maxima was too distant from the maxima of AmB.

With the aid of the above described chromatographic method several minor components can be detected in AmB samples. To our best knowledge, the separation of these components has not been published before.

Table 8. Eluent composition (v/v) and gradient program for the HPLC analysis of amphotericin B and minor components

Eluent composition		A (%)	B (%)
Acetonitrile		32,4	50,4
Methanol		12,6	19,6
Buffer Na-Ac EDTA (pH 5)		55,0	30,0
Gradient program			
Time (min.)	Flow (ml/min.)	A (%)	B (%)
0,05	0,25	100	0
4,0	0,25	20	80
12,0	0,25	20	80
14,0	0,25	100	0
30,0	0,25	100	0

Figure 16. Heptaene and tetraene components in Fungizone by HPLC



The precise detection and identification of them is of great importance during a stability test where degradation products are formed and the amount of main (and active) antifungal is constantly changing. The new chromatographic parameters can beneficially be used during a stability test in the future.

3. Thin Layer Chromatography

a) Materials and methods

Solutions of AmB (0.5 mg/ml) were prepared in a solvent mixture composed of 30 % DMSO (analytical grade, Merck KGaA, Darmstadt, Germany), 60 % methanol (HPLC grade, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and 10 % distilled water from three different substances: (1) Vetranal - amphotericin B trihydrate Riedel-de-Haën (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); (2) freshly prepared Fungizone (5 mg/ml; Bristol-Myers Squibb, Epernon, France) and (3) degraded Fungizone solution stored at room temperature for two years. The solutions were spotted on 20x20 cm pre-coated silica gel 60 aluminium sheets (Merck, Darmstadt, Germany Art.No. 5553) – cut into 10x10 cm sections – by use of disposable 1 µl micro pipettes. TLC adsorbents were washed with methanol, dried and preconditioned by heating at 120 °C for 3 hours. Ascending development to a distance of 8 cm was performed in a previously saturated (for 20 minutes) CAMAG (Muttenz, Switzerland) chromatographic chamber. After development the plates were dried and UV active spots were detected at 366 nm. Photographic documentation was performed with a digital camera (Nikon Coolpix 5700, resolution 2560x1920 pixels, Nikon Corporation, Tokyo, Japan). Densitometric evaluation was performed with CAMAG TLC Scanner II V3.15 with CATS Version - 3.14. (CAMAG, Muttenz, Switzerland). Scanning conditions were: slit dimension, 12 x 1.2 mm; monochromator brandwith, 30 nm; light source, deuterium lamp at λ=385 nm. Chromatographic developments were performed at room temperature. The TLC sheets were dried also at the same temperature, protected from light with good ventilation to reduce the risk of the degradation of the substance.

b) Results and conclusions

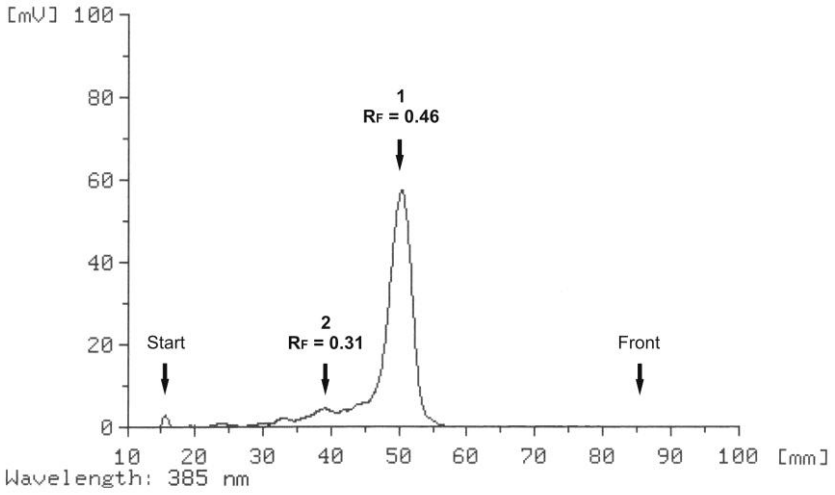
The most detailed review regarding the TLC separation of polyenes can be found in the publication by Thomas (54). Out of the 7 potential eluent systems we chose to test the ones with R_F values over 0.2 in hope of better separation and biologic detection (**Table 9.**). Because solvents or their residues can modify the microbiologic detection, eluent selection is considered to be a complex problem for direct bioautography.

Table 9. Formerly documented mobile phases tested on silica gel layers and the optimized eluent composition for TLC and direct bioautography

	Mobile-phase components	Volume ratio (v/v)	Measured R_F	Running time (min)	No. of components at 366 nm
A	Methanol – Acetone – Acetic acid	8:1:1	0.63	25	1
B	1-Butanol – Pyridine – Water	3:2:1	0.78	90	1
C	Chloroform – Methanol – Borate buffer (pH 8.3)	7:5:1	main:0.29 minor:0.21	35	2
D	1-Butanol – Ethanol – Acetone – conc. Ammonia	2:5:1:3	0.25	60	1
E	Optimized eluent: Chloroform – Methanol – Borate buffer (pH 8.3)	4:5:1	main:0.46 minor:0.31	27	2

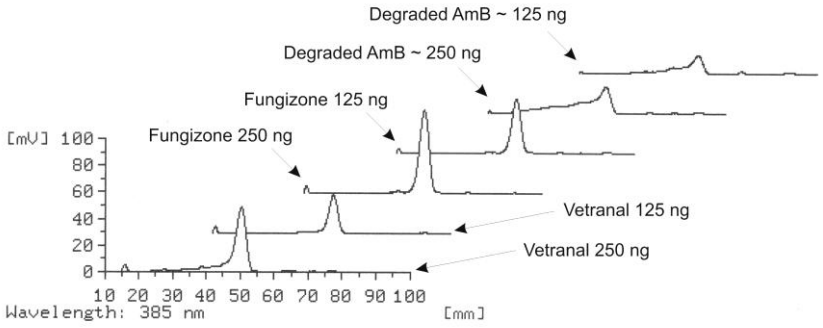
As presented in **Table 9**., with the application of eluent C two spots can be observed on the plates at 366 nm. For the better separation of the components and future identification of degradation products we optimized this eluent. Alteration of pH and ionic strength of the buffer did not affect separation, but increasing the amount of the inorganic component resulted in higher R_F values and an augmented distance between the spots. Raising the amount of methanol within the organic phase increased R_F values at the expense of the separation of the components. Our final eluent E has the advantages of a relatively fast TLC development and the use of easily evaporating organic solvents, appropriate for microbiologic detection. As shown on the densitogram (**Figure 17.**) the major component of AmB is at R_F = 0.46. Separation of minor heptaenes is relatively difficult to achieve and detection of the components is complicated, because the UV visualization of minor components can only be achieved at high concentrations (above 100 ng/spot). Unfortunately at higher concentrations the spots are overloaded and tailing is observed, thereupon the separation of the components is flawed.

Figure 17. Densitogram of amphotericin B (250 ng/spot) with main component (1) and minor component (2) at 385 nm



At concentrations of 250 - 500 ng/spot both the major and one minor component can be seen under the UV light. Our intention was to document changes in the composition of degraded solutions and also to identify degradation products in Fungizone 5 % solutions. When measuring the three different samples (analytical standard, fresh and degraded Fungizone) we had to find, that compared to the analytical standard (Vetranal) and the freshly prepared Fungizone no discrete degradation product or minor component can be separated (**Figure 18.**).

Figure 18. Densitogram of freshly prepared and degraded samples at 385 nm.



Based on the densitometric analysis (250 and 125 ng) it can be stated, that the main component of AmB has degraded because not only the area under the curve, but also the height of the peak is reduced in the densitogram of the stored/degraded samples (measurement data (n=1) presented in **Table 10.**). Probably many different products are produced which could be causing the "tail" of the peak. From this aspect the TLC method in itself is not the most suitable method for the qualitative analysis of degradation products in AmB solutions, but it can indeed be a useful marker of degradation. The question arose whether the degraded components possess any antifungal effect or not? This question can be answered with the aid of microbiologic detection, such as direct bioautography.

Table 10. Analysis of analytical standard, fresh and degraded solutions (5 mg/ml) of amphotericin B with densitometry

Sample of AmB	Peak start		Peak max		Peak end		Area
(250 ng/spot)	Distance (mm)	Height	Distance (mm)	Height	Distance (mm)	Height	
Vetranal (standard)	44.9	6.5	50.9	58.2	56.7	2.0	2571.2
Fungizone (fresh)	45.9	6.5	50.7	57.5	56.3	0.6	2375.3
Fungizone (stored/degraded)	42.8	5.0	50.7	32.2	56.3	0.1	1561.9

E. Biological methods

1. Introduction

Biological techniques are useful indicators of antimicrobial potency and degradation, resulting in loss of microbiological activity (*see illustrative figure in Appendix D.*). The activity of an antifungal or an antimicrobial substance can be determined by its inhibitory effect on microorganisms. Bioassay is used for quantification of such substances which cannot be measured adequately by chemical or physical methods and also when the reduction of antimicrobial activity could reveal changes which cannot be measured by chemical methods. Antibiotic assays are performed by agardiffusion or turbidimetric methods. In case of agardiffusion, the sample solutions are measured in cylinders cut in a seeded agar, or disks containing antibiotics are placed on the surface of a medium inoculated by a sensitive test strain. The concentration / activity of the sample can be calculated from the diameter of the inhibition zones. The parallel-line model is an analytical method described by the Ph. Eur. 6. for the bioassay of antibiotics (68). The validity of the results requires the log dose-response curve to be linear in the measured concentration range. Because the U.S., European and Hungarian pharmacopoeias recommend bioassay for the quantification of polyenes, the application of agar diffusion method seemed to be an appropriate analytical method. We observed that using the conditions proposed in Ph. Eur. 6. (indicator organism and assay medium), the reproducibility of the assay was difficult: limit of detection was relatively high; inhibition zone borders were indistinct; the agar medium filled with gas bubbles produced by the test microorganism (*Saccharomyces cerevisiae* ATCC 9763) and cracked after 24 hours of incubation. These factors lead to the inaccuracy of the assay. For these reasons we tested, which assay parameters would be the most optimal for the quantitative measurement of AmB during a stability test, where a wide concentration range is aimed to be measured.

Direct bioautography, as a post chromatographic detection for microbiologically active substances, have been used by several authors in different fields of analytical, medical or agricultural sciences. Hostettmann (69) describes bioautography methods as very convenient and simple ways of testing plant extracts and pure substances. In search of detecting new antifungal several methodologies were used and compared, for example Rahalison (70) have described the use of series of clinically important antimycotic agents during their experiments

and also Wedge and Nagle (71) have tested mixtures of structurally diverse antifungal substances (such as AmB).

2. Bioassay with agardiffusion

a) Materials and methods

In our study we compared four commonly used assay media: Mueller Hinton Agar (MHA); MHA supplemented with 2% glucose and methylene blue (MHA-GMB); Modified Antibiotic Medium 12 (MAM-12), the Ph. Eur. 6. antibiotic medium F (F-M) and a medium (MHA-E) used in our institute for bioassay (see *Appendix E*.). As indicator organisms we chose *Candida albicans* (ATCC 90028) and *S. cerevisiae* (ATCC 9763) both previously used for bioassay of AmB. The sterile, melted media prepared in volume of 250 ml were cooled to 50 °C and maintained at that temperature until dispensed. Prior to dispensing the melted agar media were inoculated with 3 ml of the adjusted suspension (absorbance = 0,1 at 600 nm) of the indicator organism which was cultivated on Sabouraud's dextrose agar (Oxoid Ltd., Cambridge, UK) for 72 hours. Colonies were washed and diluted with sterile physiological saline solution. 25 sterile antibiotic assay cylinders (diameter: 8 mm) were placed equidistance apart in sterile glass petri dishes (diameter: 250 mm). The cylinders were allocated according to a completely randomized design and were loaded with 200 µl sample solutions. AmB solutions are prepared from Fungizone (Bristol-Myers Squibb, Epernon, France) using the solvent (DMSO, Merck, Darmstadt, Germany) and the buffer (0,2M phosphate buffer) indicated in Ph. Eur. 6. for dilution of the samples. Fungizone has been used as the raw material for the nasal sprays and also as the subject for the stability test, since the pharmaceutical grade substance was inaccessible in Hungary and earlier studies also used other formulations of AmB medications. Drug dilutions used for the dose-response curve contained 60,0; 24,0; 9,6; 3,84; 1,54; 0,61; 0,25 and 0,10 µg/ml AmB. Three replications per dose were performed. A period of diffusion (2 hours at 5 °C) was used to minimize the effects of the variation in time between the application of the samples and to improve the regression slope. Afterwards the plates were incubated at 30 °C for 48 hours. To determine the dose-response curve, we photographed the plates in a dark chamber, lit with 20W light, with a digital camera (Nikon Coolpix 5700, resolution 2560x1920 pixels) while placing a 10 mm

reference scale on the agar surface. Taking three measurements across different diameters, the zones of inhibition were determined with a precision of 0,01 mm.

The applicability of the official bioassay analysis is based on the assumption that the log dose-response curve is linear in the measured concentration range. The linear regression was tested with curve fit and analysis of variance (ANOVA). A segment of the log dose-response curve was considered to be linear if $R^2 \geq 0{,}95$ and the correlation between the logarithm of the dose and the response was significant ($p \leq 0{,}05$). Calculations were performed with SPSS 14.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

c) Results and conclusions

Limits of detection and linear segments of the log dose-response curves for the examined assay media and test organism are summarized in **Table 11.** Assay media without any glucose (MHA-E and MHA) are unsuitable for bioassay with *S. cerevisiae* since no growth, thus no zones of inhibition can be detected. When media with glucose for *S. cerevisiae* was used, intense gas formation and bubbles were observed after 24-32 hours, which reduced the accuracy of determining the inhibition zones. This phenomenon is corrigible if after a 30 °C 48 hours incubation, the plates are stored at 5 °C for 24 hours, allowing the gases to escape.

Lowest limit of detection (0,25 μg/ml AmB) can be achieved using MHA-E and *C. albicans*. For the other observed media *S. cerevisiae* increases the sensitivity of the method, however using *C. albicans* longer linear segments in the log dose-response curve can be found, except in the case of MAM-12. The assay parameters suggested by the Ph. Eur. 6. (F-M and *S. cerevisiae*) give same results as employing MHA-GMB with *S. cerevisiae* (**Figure 19.).** Exchanging the test organism to *C. albicans* enables to measure an approximately 6 times wider concentration range (1,54-60,0 μg/ml), which condition is much suitable for a stability test. If a lower concentration is to be detected, the MHA-E with *C. albicans* is the most appropriate choice, since AmB can be detected between 0,25-9,6 μg/ml.

Table 11. Limits of detection and linear segments of the log dose-response curves of amphotericin B for the observed assay media and test organism

Medium code	Indicator organism	Limit of detection (μg/ml)	Linear segment of the dose-response curve (μg/ml)			R^2	p
MHA-E	*C. albicans*	0,25	0,25	-	9,6	0,967	0,003
	S. cerevisiae						
MAM-12	*C. albicans*	3,84	9,6	-	60	1	0,005
	S. cerevisiae	1,54	3,84	-	60	0,998	0,001
MHA	*C. albicans*	0,61	0,61	-	9,6	0,964	0,018
	S. cerevisiae						
MHA-GMB	*C. albicans*	1,54	1,54	-	60	0,966	0,003
	S. cerevisiae	0,61	0,61	-	9,6	0,954	0,023
F-M	*C. albicans*	1,54	3,84	-	60	0,955	0,023
	S. cerevisiae	0,61	0,61	-	9,6	0,974	0,013

Abbreviations: MHA-E: Assay media used at the University of Pécs; MAM-12: Modified Antibiotic Medium 12; MHA: Mueller Hinton Agar; MHA-GMB: Mueller Hinton Agar supplemented with 2% glucose and 0,5 μg/ml methylene blue; F-M: Antibiotic medium F in Ph. Eur. 6. For composition see *Appendix E*.

Readability of the inhibition zones is a crucial part of a bioassay analysis. If the borders of the zones are more distinct, the reproducibility of the method is better. In our study we documented and compared the zones of inhibition for each examined circumstance after 48 hours of incubation at 30 °C (**Figure 20.**). Borderlines are more diffuse when *S. cerevisiae* is used as test organism. No growth can be observed on the glucose free MHA-E medium and only slight growth is seen on the starch containing MHA medium. In case of employing the F-M medium and *S. cerevisiae* as an indicator strain (Ph. Eur. 6. conditions) a double inhibition zone is produced, which further reduces the good readability of the zones. Using *C. albicans* as the test organism, both with MHA-E and MHA-GMB media clear and distinct zones of inhibitions were documented.

Figure 19. Linear segments of the dose-response curves for four selected bioassay conditions of amphotericin B.

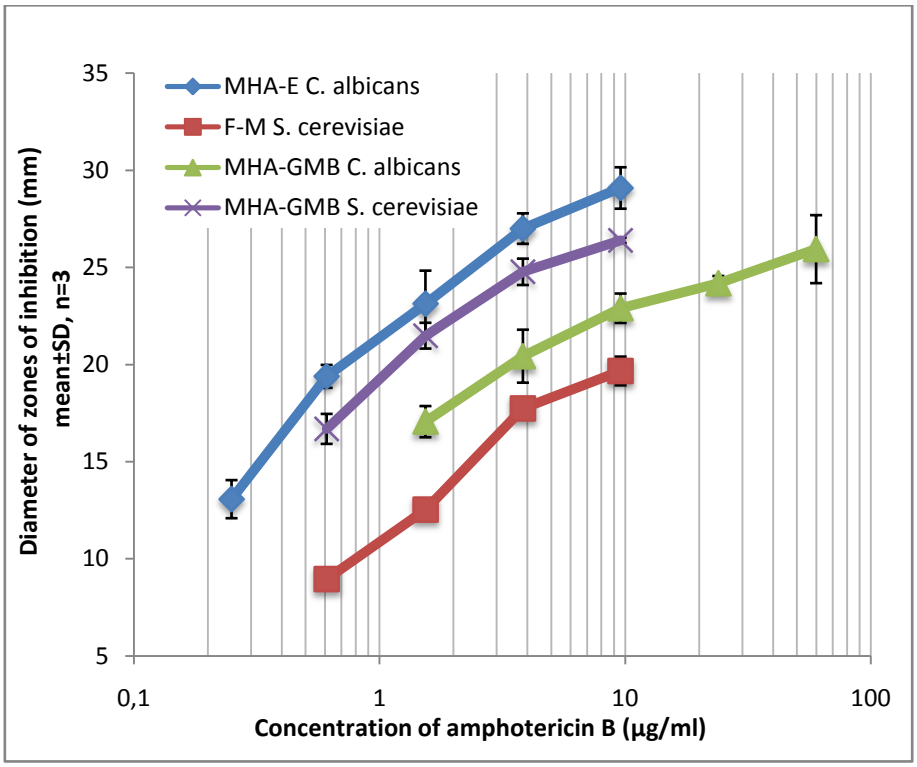
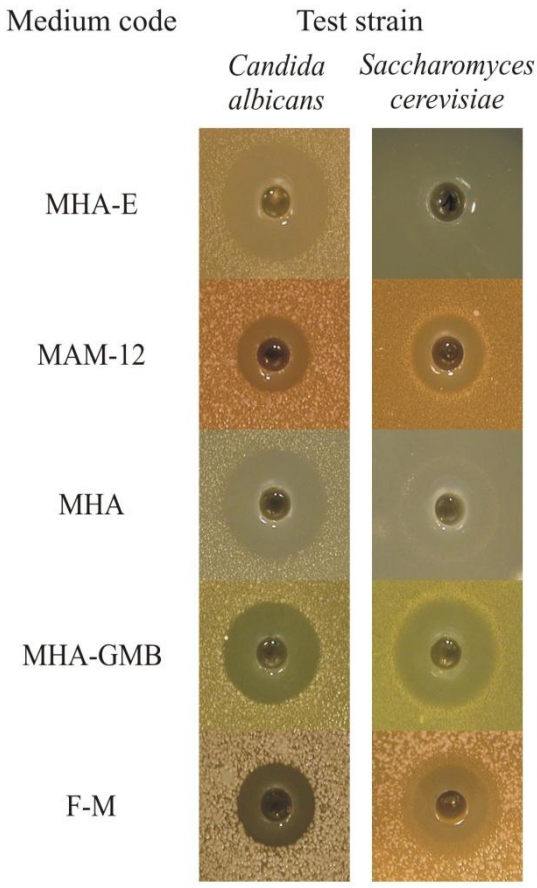


Figure 20. Zones of inhibition of amphotericin B for the studied assay media and test organism photographed after 48 hours of incubation at 30 °C.



Abbreviations: MHA-E: Assay media used at the University of Pécs; MAM-12: Modified Antibiotic Medium 12; MHA: Mueller Hinton Agar; MHA-GMB: Mueller Hinton Agar supplemented with 2% glucose and 0,5 µg/ml methylene blue; F-M: Antibiotic medium F in Ph. Eur. 6. For composition see *Appendix E*.

Our analytical method was validated for the best assay condition found (MHA-GMB and *C. albicans*) according to the ICH guideline (72). Three concentration levels were tested (low, middle and high). At each level, samples were prepared in triplicate and results were analyzed for the same day to test the repeatability (intra day precision). Accuracy was expressed as percentage of the observed and the theoretical concentration. To test the precision variation within different days (intermediate precision) measures were repeated on three consecutive weeks. Precision data is expressed in percentage relative standard deviation (R.S.D). According to our tests the limit of detection (LOD) for the most optimal condition

was 1,54 µg/ml and the limit of quantification (LOQ) was 15 µg/ml. In **Table 12**. the results of the method validation are shown. The accuracy of a quantitative bioassay method unfortunately can't be compared with the precision of other instrumental methods such as HPLC. Although according to the validation data the accuracy is within the ±5 % range at all measured concentrations, the higher deviation at low concentration implies that AmB could be more accurately measured at higher concentrations.

Table 12. Results of method validation

Theoretical concentration (µg/ml)	Measured concentration (µg/ml) (mean n=3)	Accuracy (%)	Precision	
			Repeatability R.S.D. (%)	Intermediate precision R.S.D. (%)
60,00	58,20	97,00	12,93	9,98
30,00	30,06	100,21	10,59	5,60
15,00	15,63	104,19	6,32	24,01

Our aim was to find the most adequate conditions for bioassay quantification of AmB during a stability test where a relatively wide range of concentration change can be expected. Thus our objectives were to find the parameters where the linear segment of the log dose-response curve is the longest, the limit of detection is the lowest and the borders of inhibition zones are distinct. In our study we evaluated five commonly used assay media and two test microorganisms for the quantitative determination of AmB in a water base solution made from Fungizone.

The assay parameters (F-medium with *S. cerevisiae* as test organism) recommended by the Ph. Eur. 6. were less sensitive and were only applicable for the measurement of a narrow concentration range. The borders of the inhibition zones were indistinct and during the incubation the medium filled with gases which made the determination of the concentration inaccurate. According to our study Mueller Hinton agar supplemented with 2 % glucose and 0,5 µg/ml methylene blue, inoculated with *C. albicans* (ATCC 90028) proved to be the most suitable bioassay parameters for the measurement of AmB during a stability test, where a

relatively wide concentration change is to be measured. The linear segment of the log dose-response curve was between 1,54-60,0 μg/ml and the inhibition zones could be read easily and accurately.

3. Direct bioautography

a) Materials and methods

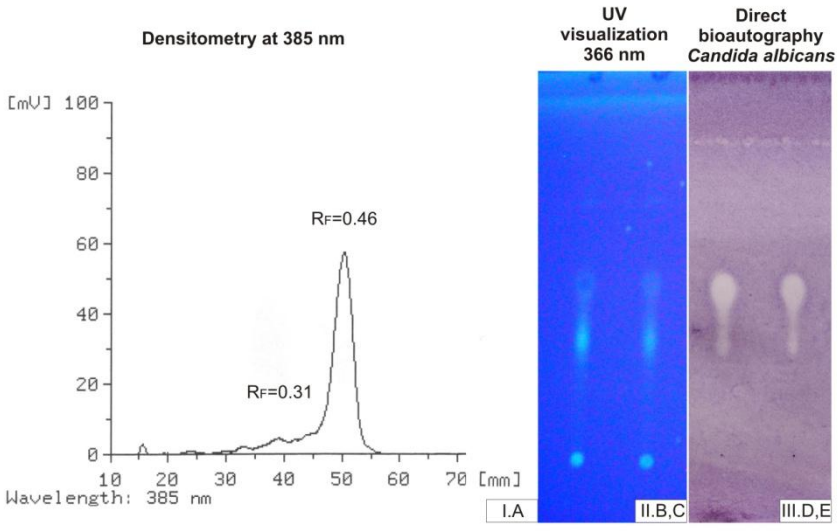
Candida albicans (ATCC 90028) and *Saccharomyces cerevisiae* (ATCC 9763) fungus strains were cultivated on Sabouraud's dextrose agar (Oxoid Ltd., Cambridge, UK) for 72 hours. One hundred millilitres of Mueller–Hinton broth (Difco Laboratories, Detroit, Michigan, USA) supplemented with 2 % glucose was inoculated with the test organism and was cultivated for 16 hours at 30 ˚C in an orbital shaker incubator at a frequency of 300 rpm (New Brunswick Scientific Innova 40, New Brunswick, NJ., USA). To achieve homogenous immersion suspensions, the optical density of the suspensions was set to 0,5 at 600 nm (in 1 cm light path) with fresh nutrient broth. The plates were immersed in the microbial suspension for 15 seconds, placed in a water vapour-chamber for incubation at 37 °C. After 5 hours the viability of microbes was detected with an aqueous solution (2.5 mg/ml) of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St Louis, MO, USA) containing 0.1% Triton X-100 (Sigma). The plates were dipped into the MTT solution for 60 seconds and dripped and dried for 30 seconds. The second incubation period lasted for 12 hours under same conditions. At the end of the experiment plates were washed with 70 % ethanol, dried at room temperature and photographed under white light (20 W). Direct bioautographic detection process was performed according to the principals described by Botz et al. (73) (74).

b) Results and conclusions

Directly after the TLC development the layers were dried at room temperature for 2 hours so the solvents could evaporate. Afterwards microbiologic detection was performed as described previously. The suitability of the two most commonly used test organisms *Candida*

albicans (ATCC 90028) and *Saccharomyces cerevisiae* (ATCC 9763) were tested. For the detection of AmB *Candida albicans* gave more sensitive detection results and also more distinct zones of inhibition were observed on an evenly blue/purple layer colour. The viability of the test fungus is a crucial point of direct bioautography. It is important that the fungus suspension is grown to the log phase. That the optimal time of growth of the cultures in Mueller–Hinton broth supplemented with 2 % glucose was after 16 hours of incubation.

Figure 21. Comparison of the results of densitometry, UV visualization and direct bioautography of freshly prepared amphotericin B solutions



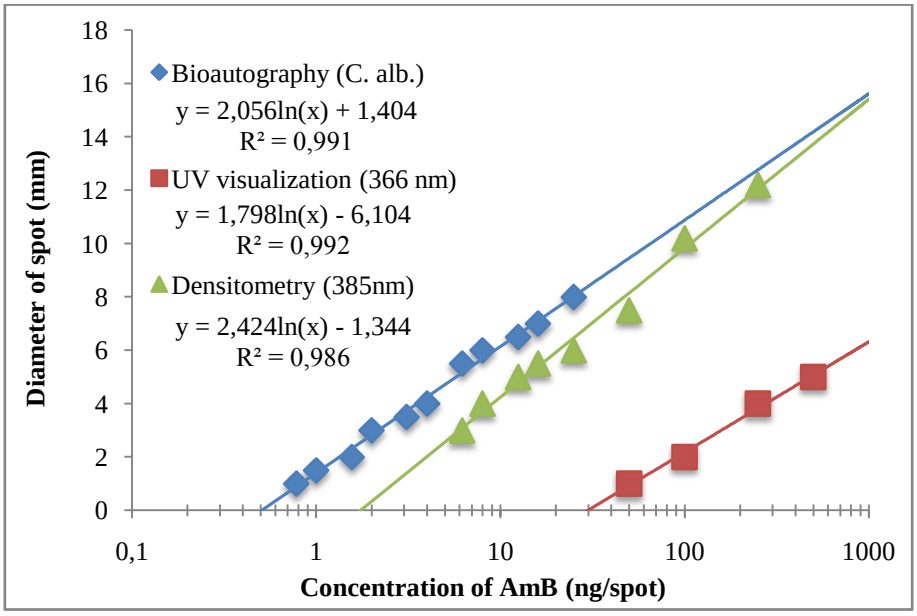
I. A.: 250 ng/spot; II.B.: 500 ng/spot, C.: 250 ng/spot; III.D.:25 ng/spot, E.:12.5 ng/spot

Just as for the UV detection direct bioautography also has its optimal concentration range for the measurement of the components. Since the microbiologic measurement is more sensitive, as little as 25 ng of AmB per spot is optimal for the observation of the minor component (R_F=0.31) **(Figure 21.)**. At higher concentrations the spots cannot be differentiated partly because during dipping of the layers into the inoculated Mueller-Hinton broth access active ingredient is washed over the layer from the overloaded spots, in this way spoiling the microbiologic detection. At lower concentrations the minor component cannot be detected presumably because its amount is below its minimal detectable concentration.

During our experiments we observed that microbiologic detection is conspicuously more sensitive than UV visualization where at least 50 ng of AmB is necessary (**Figure 22.**). The densitometer can detect as little as 4 ng of AmB, but peaks can be integrated when the

amount spotted is over than 25 ng of AmB. As for direct bioautography even 0.8 ng AmB is detectable, thus 0.8 µg/ml AmB solutions can accurately be measured.

Figure 22. Detection limits of amphotericin B with three different methods

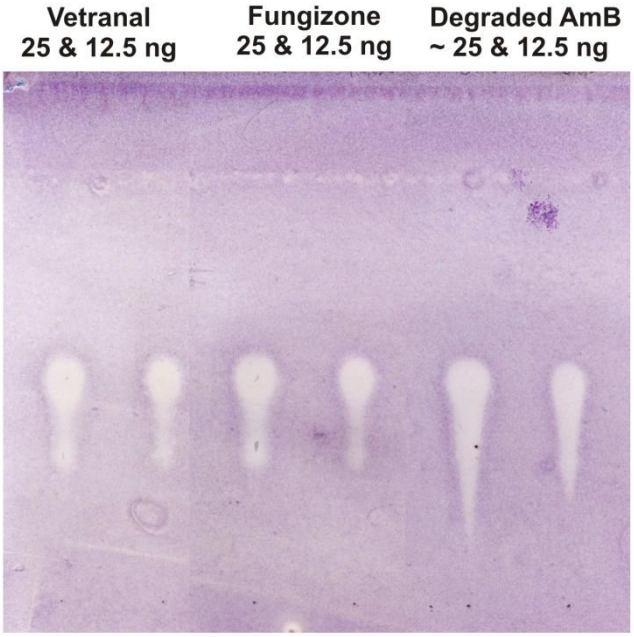


Since DMSO could influence the chromatographic separation and might exert antifungal effect at certain concentrations, we tested each parameter and came to the conclusion that neither the R_F value and spot form, nor the fungal cell growth is influenced by the amount of DMSO used in our experiments.

Our aims were to separate and detect the microbiological activity of main and minor components in fresh and degraded AmB solutions which were used as nasal sprays in a clinical study. We had to face a complex analytical problem because TLC of polyenes is poorly documented, direct bioautography of amphotericin B has not been published before and degradation of the observed substance makes the separation and the microbiologic visualization troublesome. With the aid of our optimized eluent (Chloroform – Methanol – Borate buffer pH 8,3; 4:5:1) two components can be separated on silica gel layers in AmB samples. The observed components both have antifungal effect, but since the minor component is present only in small amount, it can be visualized only when higher sample concentrations are used. According to our experiments a mixture of degradation products are

produced during storage of AmB solutions (**Figure 23.**). The degradation does influence chromatographic behaviour but does not alter the antifungal effect strikingly.

Figure 23. Direct bioautography of freshly prepared (Vetranal and Fungizone) and degraded samples of amphotericin B solutions detected with *Candida albicans*.



It can be stated that direct bioautography proved to be a sensitive method with the detection limit of 0.8 ng/spot. Both with Vetranal and Fungizone samples same result were produced in the observed concentration range (0.5-1000.0 ng/spot) in case of TLC separation and also with microbiologic detection with *C. albicans*. We believe that with the above described method the examination of clinical study test samples will be complemented with information regarding the amount and activity of degradation products. In our judgment direct bioautographic detection method can be useful for future measurements since apart from being sensitive for a commonly used polyene antifungal of clinical practice, it is relatively fast, cheap and easy to perform.

F. Conclusions for the analysis and stability testing of amphotericin B preparations

As described in the second part of my thesis, finding the most suitable analytical method for the measurement of AmB is a difficult task. The combined application of biological and chemical methods enables us to document the concentration and activity changes in AmB samples most precisely. With the aid of an accurate HPLC analysis and a sensitive bioassay, reliable information can be gathered during a future stability test.

Further improvement of our methods would be the elaboration of a more sensitive HPTLC separation followed by direct bioautographic detection. It is very likely, that quantitative measurements can be achieved if the method is further improved based on the previous experience gained during the described bioassay measurements.

In case a new stability test would be required, it would definitely be beneficial to document colloidal changes in the solutions also. This would contribute to the better understanding of physico-chemical changes occurring in water based solutions and also might give answers to the puzzling phenomena of temporary increase of antifungal activity.

The questions razed and the problems solved during my analytical work all originated from a practical pharmaceutical task. Apart from the fact, that I had the chance to get acquainted with a variety of useful methods, the complexity of the analysis of polyenes is summarized in a way that might also be beneficial to other researchers in the fields of analytical sciences.

VI. Final general summary

Various topics from different fields of pharmaceutical sciences have been discussed in the thesis. Certainly a drawback of such diversity can be that no single project was specified in full detail, on the other hand, as an advantage, the thesis introduced a pharmaceutical approach which evolved during my work and my personal professional ideology.

Earlier clinical studies regarding the efficacy of antifungal treatment for chronic rhinosinusitis were summarized and evaluated. Emphasis was laid on pharmaceutically important factors such as stability, compliance and study design. Despite the heterogeneity of the published papers with contradictory results it was concluded, that chronic rhinosinusitis with nasal polyposis has an unclarified pathogenesis and the effectiveness of local antifungal therapy is far from clear. Nasal sprays were considered to be the most suitable form of drug delivery most importantly because of the convenience for patients, thus improving the adherence rate. Spray solutions were prepared under aseptic conditions by the hospital pharmacy. Glucose was omitted from the solutions because of the assumption that it might facilitate the growth of fungi. Patient adherence was relatively good. Even though a moderate decline was seen from month to month, at the end of the study the overall adherence was notably above the most commonly documented cut-off point for adherence of 80 %. The most common barrier to adherence was motivational. The incidence and also the regularity of side-effects were higher in the AmB group than in the placebo group.

For the evaluation of long-term efficacy of amphotericin B treatment, a prospective randomized placebo controlled trial was conducted, involving 33 patients. Patients with nasal polyposis were operated on with an endoscopic technique and were treated with a nasal spray containing 5 mg/ml amphotericin B, while the placebo group received a nasal spray lacking amphotericin B. We evaluated our results with the aid of a modified Lund-Mackay CT score, the SNAQ-11 test, a quality of life test and endoscopy. Our results lead to the conclusion that the administration of amphotericin B nasal spray to patients operated on for nasal polyposis does not give rise to a significant alteration in either CT score, clinical symptoms, or quality of life.

The second part of the thesis summarizes and compares chemical (spectrophotometry, HPLC) and microbiological (bioassay, direct bioautography) methods for the quantitative measurement of amphotericin B. Optimized HPLC, TLC, bioassay and direct bioautography

methods were developed for the qualitative and quantitative measurement of amphotericin B. Several important observations were made regarding these optimized techniques. The stability test of amphotericin B nasal spray solutions was performed. The two detection methods applied (spectrophotometry and bioassay) showed strikingly different results. According to the chemical analysis the samples are considered relatively stable under all observed conditions, while bioassay showed complete loss of antifungal activity after 35 days of storage at room temperature and notable decrease was be observed at 4°C. We estimated the shelf life of the glucose-free solutions, being stored at 4°C, to be 30 days in accordance with our bioassay results. With the aid of our improved HPLC method several minor components can be detected in AmB samples. Earlier TLC methods for amphotericin B were tested and a new eluent system was documented. Although this TLC method in itself is not the most suitable method for the qualitative analysis of degradation products in AmB solutions, but it can indeed be a useful marker of degradation. Direct bioautography further improved the sensitivity of the method. Because the bioassay parameters recommended by the Ph. Eur. 6. were less sensitive and were only applicable for the measurement of a narrow concentration range, an optimized method was improved capable of the quantitative measurement of amphotericin B at the concentration range of 1,54-60,0 μg/ml.

All these observations contribute to the better – although still debatable – understanding of the role of local antifungal therapy in chronic rhinosinusitis and certainly the summarization and improvement of analytical methods will be highly beneficial for future clinical studies.

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IX. Appendix

A. Number of organisms recovered from patients with CR (N=210) and percentage of patients colonized with the species

Acremonium	5	(2.4%)
Alternaria	93	(44.3%)
Arachniotus citrinus	3	(1.4%)
Arthrographis kalrae	1	(0.5%)
Aspergillus	62	(29.5%)
A flavus	8	
A fumigatus	17	
A glaucus	6	
A nidulans	1	
A niger	5	
A terreus	2	
A versicolor	15	
A versiforme	1	
Aspergillus species*	7	
Aureobasidium	8	(3.8%)
Beauveria	2	(1.0%)
Bipolaris	2	(1.0%)
Candida	45	(21.4%)
C albicans	31	
C krusei	1	
C lipolytica	1	
C lusitaniae	1	
C parapsilosis	9	
C tropicalis	1	
Candida species.*	1	
Chaetomium	3	(1.4%)
Chryosporium	2	(1.0%)
Cladosporium	82	(39.0%)
Cryptococcus	4	(1.9%)
C albidus	1	
C laurentii	2	
Cryptococcus species*	1	
Curvularia	2	(1.0%)
Epicoccum	12	(5.7%)
Exophiala jeanselmei	2	(1.0%)
Fusarium	34	(16.2%)
Geotrichum	10	(4.8%)
Gliomastix	1	(0.5%)
Monilia	3	(1.4%)
Mucor	4	(1.9%)
Nigrospora	1	(0.5%)
Oidiodendron	1	(0.5%)
Paecilomyces	5	(2.4%)
Papularia	4	(1.9%)
Penicillium	91	(43.3%)
Phoma	2	(1.0%)
Pithomyces	14	(6.7%)
Pseudallescheria boydii	1	(0.5%)

Rhinocladiella	3	(1.4%)
Rhizopus	5	(2.4%)
Rhodotorula	4	(1.9%)
R minuta	2	
Rhodotorula species*	2	
Saccharomyces cerevisiae	1	(0.5%)
Sagrahamala	1	(0.5%)
Scolecobasidium	1	(0.5%)
Scopulariopsis	3	(1.4%)
S brumptii	1	
Scopulariopsis species*	2	
Trichoderma	8	(3.8%)
Trichophyton	2	(1.0%)
T rubrum	1	
Trichophyton species*	1	
Trichosporon beigelii	1	(0.5%)
Ustilago	13	(6.2%)
Total No. of organisms		**541**

*subclasses not available

(from The Diagnosis and Incidence of Allergic Fungal Sinusitis, J. U. Ponikau et al in Mayo Clin Proc. 1999;74:877-884)

B. Scoring systems of outcome parameters for the clinical study

1. Modified Lund–Mackay CT evaluation score system

Scoring
0 = Not opacified 1 = Less than 1/3 opacified 2 = Between 1/3 and 2/3 opacified 3 = More than 2/3 opacified, but still air-containing 4 = Complete opacification (no air)
Maximum score : 5x4x2=40 Each side was separately evaluated for opacification of the maxillary, anterior and posterior ethmoideal, sphenoidal and frontal sinus.

2. SNAQ-11 questionnaire relating to sinus symptoms questions

Nasal obstruction Feeling of nasal fullness, sneezing Facial pain, pressure feeling Anterior nasal discharge Posterior nasal discharge Sneezing Cough Diminished smell Headache Ear pain, pressure feeling in the ear Sleeping difficulties, daytime sleepiness Evaluation 0 = No symptom 1 = Very mild symptom 2 = Mild symptom 3 = Moderate symptom 4 = Serious symptom 5 = Extremely serious symptom Scores to questions 1 and 2 should be multiplied by 3, and to question 3 by 2; the maximum available score is 80.

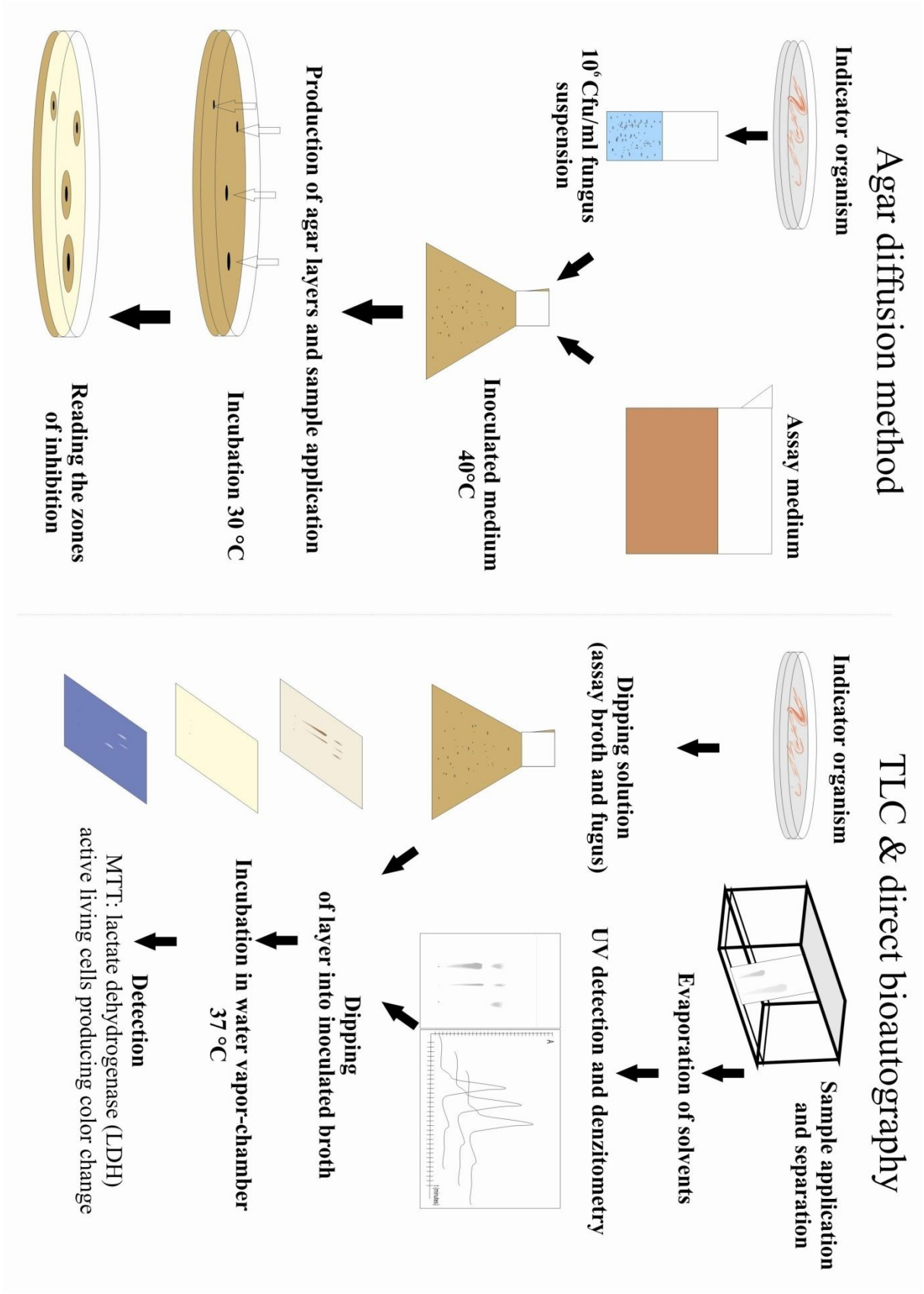
3. Quality of life evaluation score system

Subjective evaluation on 7-point scale. (0: not troubled by nasal symptoms 6: extremely troubled by nasal symptoms)
Questions Regular activities at home and at work? Recreational activities? Tiredness and/or fatigue? Sleep? Thirst? Feeling irritable? Maximum score: 36 points

C. Questions of the modified brief medication questionnaire for patients using amphotericin B nasal sprays.

Type of screens and questions
A. Regimen screen
A1. On how many days did you use the spray?
A2. How many times per day did you use it?
A3. How many doses did you apply each time?
A4. How many times did you miss using the spray?
B. Belief Screen
B1. How does this medicine work for you?
B2. Does the medication bother you in any way?
C. Recall Screen
C1. Did you ever forget to use the spray?
C2. Was it troublesome for you to use the spray twice a day?
C3. Do you feel, that you take so many medications that it is difficult for you to comply with the instructions?
D. Access Screen
D1. Did you have problems with getting the refills in time?
D2. Has it ever happened, that you ran out of spray solution earlier than the next visit due?
D3. Did the sprays work properly?

D. Comparative demonstration of agardiffusion and direct bioautographic measurements



E. Composition of the examined media for the bioassay of amphotericin B

Assay medium	Code	Composition	
University of Pécs Bioassay Medium	MHA-E	$Na_2HPO_4x10H_2O$[1]	4,0 g
		NaCl[2]	5,0 g
		Lab-Lemco[3]	5,0 g
		Peptone (pepsin digested meat)[1]	10,0 g
		Agar[4]	20,0 g
		Distilled water	1000 ml
Modified Antibiotic Medium 12 [4]	MAM-12	Yeast extract[1]	7,0 g
		Peptone (pepsin digested meat)[1]	7,0 g
		Glucose[2]	15,0 g
		Agar[4]	20,0 g
		Distilled water	1000 ml
Mueller Hinton Agar	MHA	Bio Rad MHA 84888[5]	35,0 g
		Distilled water	1000 ml
Mueller Hinton Agar supplemented with 2% glucose and 0,5 µg/ml methylene blue [5, 6]	MHA-GMB	Bio Rad MHA 84888[5]	35,0 g
		Glucose[2]	20,0 g
		Methylene blue	0,5 mg
		Distilled water	1000 ml
Ph. Eur. 6. medium F [7]	F-M	Peptone (pepsin digested meat)[1]	9,4 g
		Yeast extract[1]	4,7 g
		Lab Lemco[3]	2,4 g
		NaCl[2]	10,0 g
		Glucose[2]	10,0 g
		Agar[4]	20,0 g
		Distilled water	1000 ml

[1] Reanal, Budapest, Hungary; [2] Hungaropharma, Budapest, Hungary; [3] Oxoid Ltd, Cambridge, UK; [4] Merck, Darmstadt, Germany; [5] Bio-Rad, Hercules, USA

X. Publications, posters and oral presentations

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2. **Fittler A**., Mayer A., Kocsis B., Gerlinger I., Fónay F., Botz L.: Amfotericin B orrspray oldat stabilitásvizsgálata biológiai és kémiai detektálással. Acta Pharmaceutica Hungarica *77*, 159-164 (2007)
3. Gerlinger Imre dr., **Fittler András**., Mayer Anna., Fónay Fruzsina dr., Pytel József dr., Botz Lajos dr. Amphotericin B tartalmú orrspray posztoperatív alkalmazása orrpolypózissal járó krónikus rhinosinusitis eseteiben – megelőzhető-e a recidíva? Fül-, Orr-, Gégegyógyászat *53(4),* 150-159 (2007)
4. **Fittler A**., Matus Z., Kocsis B., Botz L. Amfotericin B tartalmú készítmények kvantitatív analízisének kémiai és mikrobiológiai vonatkozásai Acta Pharmaceutica Hungarica *78*. 69-74. (2008)
5. Gerlinger I., **Fittler A**., Mayer A., Patzkó Á., Fónay F., Pytel J., Botz L. Amphotericin B-tartalmú orrspray posztoperatív alkalmazása orrpolyposissal járó krónikus rhinosinusitis eseteiben. Megelőzhető-e a recidíva? Orvosi Hetilap *149(37),* 1737-1746. (2008)
6. Imre Gerlinger; **András Fittler**; Fruzsina Fónai; Ágnes Patzkó, MD; Anna Mayer; Lajos Botz, PhD: Postoperative application of amphotericin B nasal spray in chronic rhinosinusitis with nasal polyposis, with a review of the antifungal therapy. European Archives of Oto-Rhino-Laryngology *266(6),* 847-855 (2009). (IF:0,843/2008)
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8. **András Fittler**, Béla Kocsis, Imre Gerlinger, Lajos Botz: Optimization of bioassay method for the quantitative microbiologic determination of amphotericin B. Mycoses *53(1),* 57-61 (2010) (IF: 1.529/2008) Times cited:1
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Multicenter Randomized Controlled Study), The Laryngoscope *120(1),* 210-212 (2010) (IF:1,877/2008)

10. **András Fittler**, Béla Kocsis, Zoltán Matus, Lajos Botz. A sensitive detection method for the thin layer chromatography of amphotericin B. Journal of Planar Chromatography *23(1),* 17–21 (2010) (IF:0,982/2008)

Posters

1. **Fittler A.**, Mayer A., Botz L., Kocsis B., Gerlinger I.: Amphotericin B tartalmú vizes oldat stabilitásvizsgálata: biológiai és kémiai vonatkozások Gyógyszerkutatási Szimpózium, Debrecen, 2006. november 24-25.
2. Tóvölgyi Zs., **Fittler A.**, Gerlinger I. , Botz L.: Beteg-együttműködés orrpolyposisban szenvedő betegek klinikai vizsgálata során. Gyógyszerkutatási Szimpózium, Szeged, 2007. november 9-10.
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4. Matus Z., **Fittler A**., Kocsis B., Botz L. Chemical and microbiological aspects of the quantitative analysis of amphotericin B. Is HPLC in itself enough for quantification? 14th International Symposium on Separation Sciences NEW ACHIEVEMENTS IN CHROMATOGRAPHY September 30.-October 3. 2008. The Zora Hotel, Primošten Croatia
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6. Matus Z., **Fittler A**., Kocsis B., Botz L. Chemical and microbiological aspects of the quantitative analysis of amphotericin B. Is HPLC in itself enough for quantification? 8th Balaton Symposium on High-Performance Separation Methods and 15th International Symposium on Separation Sciences Hotel Azúr, Siófok, 2009. szept 2-4
7. **András Fittler**, Lajos Botz. Clinical and analytical aspects of the improvement of a local antifungal preparation for the treatment of nasal polyposis. 3rd BBBB-Bosphorus International Conference on Pharmaceutical Sciences, Antalya-Turkey 26. October 2009.

8. **Fittler András**, Botz Lajos, Intranazális gombaellenes készítmény fejlesztésének klinikai és analitikai vonatkozásai. Congressus Pharmaceuticus Hungaricus XIV. Budapest 2009. október 13-15.

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2. **Fittler A.**: Amphotericin B tartalmú orrspray orrpolip kezelésére.
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5. Gerlinger I., **Fittler A**., Mayer A., Fónay F., Pytel J., Botz L.: Amphotericin B orrspray alkalmazása krónikus rhinosinusitis orrpolyposissal járó eseteiben – megelőzhet-e a recidíva? Magyar Fül-, Orr-, Gége és Fej-, Nyaksebész Orvosok Egyesületének Tudományos Ülése Budapest 2008. április 25
6. **András Fittler**, Zoltán Matus, Béla Kocsis, Lajos Botz: Chemical and microbiological aspects of the quantitative analysis of amphotericin B. 9th Symposium on instrumental Analysis, Pécs 2008. június 30-július 1
7. **Fittler András**, Egy polién antifungális vegyület analízisének komplexitása. IX. Clauder Ottó Emlékverseny Budapest, 2009. április 23-24.
8. **Fittler András**, Amfotericin B orrpolyposisban történő alkalmazhatóságának kritikai értékelése. Az MTA Gyógyszerésztudományi Osztályközi Komplex Bizottságának ülése, Budapest 2009. április. 29.

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